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(54) Title: BIOSPECIFIC COMPLEX ISOLATION AND MEASUREMENT USING HAPTEN ELUTION FROM ANTI–HAPTEN ABSORBENTS

(57) Abstract

Disclosed are sensitive methods of measuring antibodies, antigens or nucleic acid molecules present in a biological sample. The methods comprise the formation of an immune complex comprising an antibody/antigen and a second, labeled antibody that immunoreacts with the antigen and a third, hapten–conjugated antibody that immunoreacts with the antigen. The complex may then be captured by an anti–hapten antibody affixed to a solid substrate and subsequently selectively eluted by an excess of hapten. The method results in detection of antibodies and antigens that are present at below background levels in standard solid phase immunoassays.

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DESCRIPTION

BIOSPECIFIC COMPLEX ISOLATION AND MEASUREMENT USING HAPTEN ELUTION FROM ANTI-HAPTEN ABSORBENTS

BACKGROUND OF THE INVENTION

The present application is a continuation–in–part of co–pending U.S. Provisional Patent Application Serial No. 60/029,501 filed October 29, 1996. The entire text of the above–referenced disclosure is specifically incorporated by reference herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of the immunodetection of antibodies, antigens or nucleic acid molecules present at low concentrations. This includes the fields of the detection of anti-toxin antibodies and anti-disease marker antibodies in medical and veterinary diagnostic techniques.

2. Description of Related Art

Several decades ago, it was learned that plastic surfaces bound proteins, nucleic acids and lipids tightly. Because these surfaces could be produced in any desired format, they have become the basis for a large number of solid-phase assays of specific biomolecules of interest. These assays took advantage of the simplicity of separation of the plastic solid phase from the solution phase reagents.

Simultaneously with the development of these *in vitro* assays on solid phases, a wide variety of reagents have been developed for measurement of specific molecules present in relevant viruses, bacteria, and animal and plant cells. This has included the molecules associated with normal development or disease processes (e.g. receptors and/or surface antigens on viruses and bacteria, cancer-associated antigens, toxic proteins produced by bacteria or fungi, nucleic acids coding for distinct toxins, etc.). The technology developed for these applications has included preparation of specific reagents (antibodies, ligands which bind to specific receptors, DNA probes, etc.) labeled with specific indicator ligands including radioactive atoms, biotin, fluorescent molecules (including fluorescein, rhodamine, etc.), and enzymes yielding colorimetric, fluorescent or luminescent products which could be bound to these reagents.

In vitro assays developed to date have combined the use of solid phases to capture specific molecules, and accomplished their detection using the indicator systems listed above. Assays performed on cells or viruses have used detection of bound ligands to identify the presence of specific molecules of interest by their appropriate labels, identified using microscopic techniques including visualization of the amount of one or more label bound to the cells identified by the specific color produced or fluorescent tag, for example. Fluorescence can also be used in a technique known as cell-sorting in which the amount of fluorescent tag bound per cell can be quantitated. In such cases, it is also possible to isolate the cells producing specific molecules by identifying the cells containing a defined amount of receptor and physically separating them from a flowing stream.

There are several important limitations to the techniques developed to date. First of all, *in vitro* immunoassays and DNA/RNA assays typically identify only two reactive sites on a biological macromolecule or other complex such as a bacterium or virus. That is because the assays developed to date depend on a single step of physical separation and identification. In this step, one of the reactive sites would be used for 'capture' of the complex onto the solid phase, and the other site would be used for the measurement of the amount of bound complex. This approach is also known as a 'capture-tag' or 'captag' or 'sandwich' immunoassay, and in its general form it is by definition only able to identify two reactive sites (which may be identical in their reactivities, as is the case for molecules repeated many times on the surface of a bacterium or virus). If one wishes to identify additional reactive sites on the same entity, it is necessary to isolate the cell or virus and perform multiple distinct assays. At the end of the analysis, it is not possible to unambiguously state that all reactivities identified were present on the same specific cell.

The identification of several specific antigens present on a bacterial surface can be important in categorizing and assessing the pathogenesis of bacteria or the potential for protection from specific vaccines. For example, Salmonella species are identified on the basis of several distinct O (somatic) and H (flagellar) antigens (Roantree, 1971). The current techniques for identification rely on isolation of a bacterial colony and use of several distinct assays for each isolated colony. It would be much more efficient to identify Salmonella species if more than one specific antigen at a time could be identified on bacteria without isolation of colonies.

This can also be important in analysis of complex molecules. One excellent example would be 20S proteasomes, which contain 14 distinct proteolytic enzyme subunits in one

complex. Most cell types apparently have the same 14 subunits, but the proteasomes found in immunological cells and interferon-induced cells have several unique subunits identified as LMP2 and LMP7, while most cells contain subunits at the same position in the complex identified as delta and MB1 (Belich and Trowsdale, 1995). Larger (26S) proteasomes also contain functionally active proteases dependent on ubiquitin and ATP, whose association with these subunits could be important in assessing function. A single sandwich-type assay or even a series of such assays for distinct antigens cannot identify the presence of combinations of these units on a single protein complex. Identification of these various subunits in a single complex could be of use in determining the nature of the complex, the cellular origin, and the ability of the complex to carry out the proteolytic function of the proteasome. Elution of intact complexes and subsequent identification of other subunits on the same entity and/or measurement of enzyme activity would be powerful adjuncts to the measurement of single pairs of associated proteasomal subunits.

There is therefore a need to go beyond the identification of single reactive components in immunoassays for complex analytical problems. A further deficiency in standard immunoassay formats, is that the analyte is destroyed in most assays. In general, this is not a problem in the assay itself but can have significant consequences for the ability to extract additional information from samples. For example, it is simpler to measure the presence of specific bacteria by ELISA (immunoassay) than it is to isolate a bacterial strain using classical microbiological isolation techniques (colony isolation and identification). This has two negative consequences, in that it is possible to measure antigens on bacteria that are no longer viable (and therefore not dangerous in food samples, etc.), and it is not possible to obtain isolates of bacteria that could be used for epidemiological surveillance when ELISA assays, which destroy bacteria, are used. It would be of significant advantage for both these limitations if bacteria could be isolated from ELISA assay plates after their presence in a particular sample is determined.

Finally, there is an artifact of immunoassays which is important in being able to identify specific reactivity, and that is the occurrence of 'non-specific binding'. This can be defined as binding irrelevant to the specific analyte being measured, which results in the presence of apparently specific signal in the absence of the analyte. This occurs especially when a very high concentration of labeled detector ligand is used in an assay, or when one is trying to measure specific reactivity in the presence of very high concentrations of similar but non-specific molecules. Examples of the first would be the use of very high concentrations of enzyme-labeled

antibody to detect low concentrations of analyte, and of the latter would be the measurement of low concentrations of specific antibody in the presence of undiluted serum. Serum immunoglobulin G (IgG) is present at a concentration of 5-10 mg/ml, and specific reactivity of interest may be due to IgG present at a concentration as low as 1 ng/ml. If a standard ELISA assay is used, which involves binding antigen of interest to a solid phase, testing diluted antiserum and then measuring bound IgG using enzyme-labeled anti-IgG, it is commonly observed that when high concentrations of serum are present the non-specific binding of serum IgG vastly exceeds the specific signal of the binding of antibody. This limits the ability to identify reactivity with antigens associated with infectious agents such as viruses and bacteria or tumors because the level of antibody (antiserum 'titer') must reach a concentration which exceeds the 'non-specific' binding of immunoglobulins to the solid phase. Approaches to reduce this non-specific binding have included using very high concentrations of protein and detergent added to the solution, and the treatment of the solid phase with 'blocking' agents which reduce this non-specific interaction. These approaches are somewhat successful in reducing non-specific binding, but they are limited in their usefulness. Improvements in the ratio of specific to non-specific binding would significantly enhance the detection of specific antibodies in serum and lead to more rapid disease diagnosis.

Hence, it is clear that a sensitive and reliable method for the measurement of an antibody response to toxins that result in potentially lethal diseases is needed. Such a method would allow the accurate detection of antibodies against various toxins which would further facilitate the production of therapeutic compositions for protection against diseases caused by the toxins as well as providing a better understanding of the toxins themselves and their potential therapeutic uses.

SUMMARY OF THE INVENTION

The present invention may be described in a broad aspect as methods of detecting and isolating a complex analyte of interest from a biological or other sample under conditions which permit the subsequent identification of other properties of the isolated analyte. The sensitivity and selectivity of the assay is based on the specific elution of a hapten conjugated immunocomplex from an antihapten solid support, using solution-phase hapten. Such elution

allows the separation of the target complex from all non-specifically bound "background" molecules that are present in a solid phase immunoassay.

The detection of the analyte is accomplished by the use of probes (antibodies, ligands capable of binding to receptors, etc) which can react with at least two reactive components present on the analyte which specifically define it, labeled with both hapten for capture onto anti-hapten antibodies present on a solid phase used for separation and a detector molecule which can be measured without destroying the analyte. A complex of the analyte with the anti-hapten antibody is then disrupted in order to separate the intact complex.

Once separated from the anti-hapten antibody absorbent, the analyte can then be selectively probed using other properties of interest in the complex. For example, if viable bacteria were separated from the solid phase, then they would be able to grow in bacterial culture media; if viable viruses were isolated, then they would be able to infect and lyse cells unless their receptors were all blocked with antibody. If an immune complex consisting of fluorescein-labeled antibody to one antigen and enzyme-labeled antibody to another antigen on a complex analyte was then mixed with a third antibody reactive with the analyte and labeled with biotin, this would be able to bind to a streptavidin-coated solid phase which would then also bind the enzyme if it was present on the same analyte. Because the labeled analyte which will be specifically bound and eluted from the anti-hapten absorbent and have bound enzyme will almost exclusively be the analyte of interest, non-specific binding will be negligible on a second solid phase used to capture the same analyte using the distinct property.

A biological sample of the invention may be a serum sample or a tissue sample, including tissue of an organ such as a liver, lung, etc., or it may be a sample of a biological fluid such as serum, saliva, semen, sputum, tear, urine or any other biological sample obtainable from a human or veterinary patient. It may also be a food or other environmental sample prepared in the form of an extract. A preferred application of the present invention is the detection of specific antibodies such as antibodies against bacteria, viruses, toxins or other disease-associated antigens such as a cancer antigen, to determine whether a subject has been exposed to such agents. The present invention provides therefore, a sensitive alternative to present antiviral antibody testing for both diagnostic and blood supply safety applications. While such a method may often be used to determine only whether such an antibody is found in the sample or not, the methods may also be used to quantitate the antibody of interest in a sample. It is also an aspect of the present invention that two or more antibodies may be tested from a single sample by the use of a

plurality of matrices combining various hapten/anti-hapten pairs and various anti-Ig antibodies, by a modification of the approach outlined below for the determination of multiple toxins in a single sample.

An application of the present invention, used as a method for measurement of human antibodies to disease-causing agents, is the detection of anti-toxin antibodies such as antibodies against botulinum toxin, to determine whether a subject has been exposed to such a toxin. Alternate embodiments within this field include the detection of anti-viral antibodies such as an anti-HIV antibody. The present invention provides therefore, a sensitive alternative to present HIV testing for both diagnostic and blood supply safety applications.

The invention applied to antibody testing may thus be described in a certain broad aspect as a method of detecting or quantitating a first, or test, antibody in a sample comprising the steps of: contacting the sample with a multivalent antigen immunoreactive with the first antibody; a second antibody immunoreactive with the same antigen or a different antigen present on the same analyte and conjugated to a detectable marker; a third antibody immunoreactive with the same antigen or a different antigen present on the same analyte, wherein the third antibody is conjugated to a hapten; and an anti-hapten antibody immunoreactive with a hapten and bound to a solid support; under conditions effective to form an immune complex including the antigen and the first, second and third antibodies and the anti-hapten antibody. Such conditions would typically include mixing of all three antibody species before contacting the analyte, especially when two or three of the antibodies may be reactive with the same antigen sites on the analyte. This method of the invention further comprises the steps of separating the solid support from the sample to obtain a solid support fraction; contacting the solid support fraction with hapten to elute the immune complex from the anti-hapten antibody; and detecting the eluted immune complex. The solid support may be any solid support known in the art such as a microtiter plate, a filter, polystyrene beads, magnetic beads, agarose and the like, for example. Haptens to be used in the practice of the invention include flourescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red, and o-phthaldehyde or any other detectable hapten known in the art. The method may further comprise the step of capturing the eluted immunocomplex on a second solid support bound to an anti-Ig antibody prior to detecting the eluted immunocomplex. This step may alternatively comprise capturing the immunocomplex on a solid support by the same or an alternative anti-hapten antibody.

The invention may also be described for antibody testing as a method for capturing a complex on an anti-hapten matrix which includes antigen preparations labeled with hapten and with detector molecule. The two labels are present on separate molecules, and the detector-labeled molecule can bind to the anti-hapten antibody only when the specific antibody is present. In this case, the invention does not require the availability of antibodies from other species to be used, and would have the same advantages of flexibility and hapten-specific elution of complexes.

The invention may also be described in a broad aspect as a method of concentrating an antibody in a sample by the method described in the previous paragraph and where the second solid substrate is a plastic or nitrocellulose filter.

Embodiments of the invention also include the detection and isolation of bacteria by capture of a colony by immune complex formation, or the capture of nucleic acid molecules by hybridization of a target molecule to a double labeled DNA as described herein. Detection and quantitation methods described include the RVV XA coagulation assay as well as more conventional colorimetric and enzymatic assays known in the art.

A specific embodiment of application of the present invention for analysis of bacteria would be the identification of specific serotypes. Thus, if for example the bacterium *E. coli* O157/H7 were to be incubated with fluoresceinated and RVV-XA-labeled antibody to the somatic antigen O157, and a biotinylated antibody to the flagellar antigen H7, then was bound to anti-fluorescein and eluted using fluorescein, the eluted bacteria bound to streptavidin-coated solid phases would by definition be the *E. coli* O157/H7 strain. The number of associated ligands would not have to be limited to these two. If the sample was an unknown serotype, one could add fluoresceinated and RVV-XA-labeled antibodies specific for any individual somatic (O) antigen. Once the serotype was identified, a mixture of various labeled antibodies specific for the various flagellar (H) antigens could be added labeled with immunochemically distinct haptens. These could be passed through a filter consisting of various anti-hapten antibodies in series, and the filter which trapped the RVV-XA-containing bacteria would identify the flagellar serotype.

Another example of the present invention would be the measurement of one of several related toxins present in a sample of interest. For example, there are seven known serotypes of *C. botulinum* neurotoxins defined as A-G and 5 known serotypes of *Staphylococcus aureus* enterotoxins defined as A-E. Analysis of contaminated samples generally proceeds in two

stages, with first the identification of a sample which is contaminated, and then identification of the serotype. For the first analysis, it is preferable to have reagents which can react with all serotypes, but to complete the analysis serotype-specific reagents must be used. If one were to label capture antibodies with two distinct haptens, one of which was common to all (e.g. fluorescein) and the other was specific to the toxin being analyzed (e.g. rhodamine for toxin A, Texas red for toxin B, biotin for toxin C, dansyl for toxin D, CY5 for toxin E, etc.) then the toxin complex would first be identified in the particular sample, eluted intact by use of fluorescein, and bound to a separate filter which was specific for the indicated second ligand present on the capture antibody and which identified the serotype.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWING

The following drawing forms part of the present specification and is included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to this drawing in combination with the detailed description of specific embodiments presented herein.

FIG. 1. The measurement of monovalent toxin-specific, affinity purified chicken antibodies.

FIG. 2. Flow diagram of complex formation with 3 distinct antibodies. Step 1. Fluoresceinated horse antibody (F1-Ab), RVV-XA-horse antibody (RVV-Ab) and human antibody (Hu-Ab) are mixed with toxin to yield a complex of the three antibodies bound to the toxin. Step 2. The complex is bound to an anti-fluorescein plate. Step 3. The complex is eluted with fluorescein. Step 4. The eluted comples is bound to anti-human IgG. Step 5. The plate is washed and an ELCA assay is used to detect complex bound to the anti-human IgG plate.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention provides for the first time, an immunoassay system which can be broadly applied for the detection of multiple analytes on a single biologically specific entity. In this work, the measurement of specific antibody at a much lower concentration than was previously possible and in the presence of undiluted serum is described in detail. The invention offers particular advantages in the detection of anti-toxin antibodies in human serum for example, which are often present at near, or below background levels. It would similarly be of particular value in the identification of anti-tumor antibodies, when the antigens associated with those tumors are known. The fact that the antibody levels could be assessed in the presence of very high concentrations of unreactive serum immunoglobulins was extremely valuable. It is the discovery of the present invention that the specific binding of the complex between the antibody and its antigen (toxin) in solution, with the inclusion of another (non-human) anti-toxin antibody conjugated to a hapten and to detector enzyme allows one to capture the entire immunocomplex with the use of an anti-hapten antibody bound to a solid support. After separating the solid support from the serum by centrifugation or other appropriate means, the immune complex which includes the anti-toxin antibody can be selectively eluted from the solid support with an excess of the hapten. This elution separates the desired fraction from the remaining non-specific human antibody that was bound to the solid support. This eluted immune complex can then be recaptured onto a second solid support by the use of an anti-human Ig antibody. In the practice of the invention, the only human antibody in the eluant which is associated with the enzyme label (bound to the non-human anti-toxin antibody) is the anti-toxin antibody that one is detecting or quantifying.

The present invention therefore exploits the fact that an antigen such as *C. botulinum* is multivalent, *i.e.*, has multiple epitopes that can be used to generate an antibody response. The same principle applies therefore to any other analyte with multiple reactive sites or epitopes on its surface and/or available for binding to additional specific ligands.

As used herein, the term "analyte" refers to any biologically significant entity with more than two reactive sites or properties which are of interest in accomplishing its complete analysis. These properties could include viability or infectivity (cells, bacteria, viruses), presence of a receptor which can bind a ligand, an enzyme active site to which a specific substrate or inhibitor that can be derivatized in the form of an analogue can be bound, an antigenic site to which

antibodies can be bound that identify unique structural determinants or antibody binding sites ('antibodies') whose presence is a reflection of the exposure of an individual to a given antigen or of pathological changes affecting that individual (e.g. autoimmune diseases), a nucleic acid with known unique sequences for which specific 'probes' can be prepared which are able to bind to those sequences.

The process involved in the assay is to prepare at least two probes of the 'entity' which define it as unique and to label these with at least one hapten and one detector molecule, then to mix these with the entity under conditions which permit the formation of complex. This complex is then bound to an anti-hapten absorbent. The absorbent in this case can be any surface which can be derivatized with a specific receptor for the hapten. The probes could be any lipid, carbohydrate, nucleic acid or protein molecule with the capacity to specifically bind to the entity being analyzed.

The hapten could be any molecule which can specifically derivatize the probe under consideration in the form of a stable linkage with the given probe. These linkages would be in a form which did not prevent the interaction of the probe with the entity being analyzed. Detector molecules could be any substance which can be specifically analyzed as an indicator of binding to the entity. These include but are not limited to radionuclides, fluorescers, chemiluminescers, enzymes, colored beads and the like. Preferred embodiments would be analytes which are not destroyed or whose detection method did not destroy the entity being analyzed in the course of their measurement, permitting the subsequent analysis of the entity.

The anti-hapten absorbent would as its preferred embodiment be a monoclonal or polyclonal antibody which can be prepared against the hapten and is able to bind it and subsequently release it under conditions which allow its rapid recovery for the purpose of subsequent analysis. The antibody could also be an 'engineered' antibody consisting of a single-chain molecule with specific binding properties for the hapten, or a molecule other than an antibody with the requisite binding specificity. The surfaces can include but not be limited to a non-porous plastic surface such as a microtiter plate or test tube, a flow-through device designed to simplify the handling of the sample, insoluble beads which may be large enough to be retained on a filter or may contain magnetic particles which would permit them to be retained on a magnet, or porous surfaces such as nitrocellulose, nylon or the like. The method of coupling may be passive adsorption or chemical coupling to the absorbent.

Subsequent to the binding to the anti-hapten absorbent, the complex is washed under conditions which permit the elimination of all or most 'background' binding which can be eliminated by washing, and then may be analyzed for the presence of such complex using a non-destructive measurement technique. It is then eluted using hapten under conditions which permit most or all of the entity to be removed from the absorbent and which do not interfere with the subsequent analysis of the entity. The form of the hapten used for elution can be underivatized or in a form coupled to proteins, nucleic acids, carbohydrates or the like, the choice of form being determined by efficiency of elution of the entity and lack of interference with the property being subsequently measured.

The entity is then bound to a second solid phase or analyzed in its eluate form. Binding to a second solid phase can be using ligands directed at additional reactive sites on the entity probed with specific labeled probes, or measuring properties such as viability, infectivity or the like as a reflection of specific elution.

Exemplary embodiments of the present invention use a first purified antibody against a toxin (F1-Ab) that is labeled with a hapten such as fluorescein, a second antibody that is labeled with a detectable marker such as Russell's viper venom factor X activator (RVV-XA) to produce a system that can be used in a coagulation detection system, for example, to detect a third component (Abtest), for example an antibody, bacterium, or nucleic acid sample, present in small amounts in any given sample. In one such embodiment the third component is another antibody present in, for example, a sample of human serum or plasma. In this embodiment the antigen is mixed with F1-Ab, RVV-XA-Ab and Abtest. The complex formed is:

F1-Ab*toxin*RVV-XA-Ab
*Abtest

The RVV-XA antibody is used as the basis of detection of the complex on either anti-hapten antibodies bound to a solid support to bind F1-Ab, or alternatively by anti-human antibodies bound to a solid support to detect Abtest. In the case where anti-human antibody is used, the specific capture would be very limited because of overwhelming presence of IgG in the plasma sample which would also be detected due to non-specific binding. However, if the complex is bound to an antihapten antibody, the only human antibodies that will be captured are those that are specific for the toxin presented because the anti-hapten antibody is specifically bound to the toxin to which the human antibody of interest is bound. These complexes may then be specifically eluted using the hapten to yield a population of anti-botulinum human antibodies

which may then be transferred to an anti-human immunoglobulin matrix for quantitation of antibody and toxin present. The use of RVV-XA for the quantification of an antibody complex is described in US Patent No. 4,668,621, incorporated herein by reference.

An alternative embodiment would be the analyte: Fl-toxin*Ab*RVV-XA-toxin which would be formed because the antibody to the toxin has at least two reactive sites, and the toxin molecule itself has multiple reactive sites. A complex between these reactive entities could likewise be bound to and eluted from the anti-fluorescein absorbent, yielding specific enzyme-labeled complex having specific antibody in the same fashion as the previous embodiment.

Thus the present invention provides a sensitive method for detecting multivalent complexes identifiable on bacteria, viruses, enzymes, receptors, antigens, nucleic acids and the like. This specificity is possible because the complex, when bound by the anti-hapten antibody can be washed free of irrelevant analytes and then can be selectively eluted from the anti-hapten absorbent, gently and specifically. Therefore, in the above example, after elution with excess hapten the only human antibody present and labeled with detector enzyme will be that antibody that is specific for the antigen presented. One of skill in the art would be able to apply this method for the isolation of complexes consisting of viruses, bacteria, enzymes, receptors, antigens or other analytes.

ELISA

As a part of the practice of the present invention, the principles of an enzyme-linked immunoassay (ELISA) may be used. ELISA was first introduced by Engvall and Perlmann (1971) and has become a powerful analytical tool using a variety of protocols (Engvall, 1980; Engvall, 1976; Engvall, 1977; Gripenberg *et al.*, 1978; Makler *et al.*, 1981; Sarngadharan *et al.*, 1984). ELISA allows for substances to be passively adsorbed to solid supports such as plastic to enable facile handling under laboratory conditions. For a comprehensive treatise on ELISA the skilled artisan is referred to "ELISA; Theory and Practise" (Crowther, 1995 incorporated herein by reference).

The sensitivity of ELISA methods is dependent on the turnover of the enzyme used and the ease of detection of the product of the enzyme reaction. Enhancement of the sensitivity of these assay systems can be achieved by the use of fluorescent and radioactive substrates for the enzymes. The inventor has recently developed a new assay methodology for clotting factors which involves coagulation, the enzyme-linked coagulation assay (ELCA). The assay involves

coating microtiter plates with fibrinogen and adding enzyme labeled fibrinogen in solution. When thrombin is added the fibrinogen is converted to fibrin and the solution phase labeled fibrin binds to the solid phase unlabelled fibrin (US patent number 4,668,621 incorporated herein by reference). Immunoassays encompassed by the present invention include, but are not limited to those described in U.S. Patent No. 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent No. 4,452,901 (western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

As used herein the term "sandwich ELISA" refers to an assay in which antibodies specific for the antigen of choice are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. A non-specific protein, such as BSA is often added to block the remainder of the well. Then, a test composition suspected of containing the desired antigen, such as a clinical sample, is added to the wells. After binding and then washing to remove unbound proteins, the bound antigen may be detected. Detection is generally achieved by the addition of another antibody, specific for the desired antigen, that is linked to a detectable label. Detection may also be achieved by the addition of a second antibody specific for the desired antigen, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label. In the current invention, the preferred form of 'sandwich ELISA' is the formation of a complex with hapten- and detector-labeled antibodies and binding of the same onto a solid phase consisting of anti-hapten absorbent.

The sandwich ELISA may also be practiced by immobilizing the antigen onto the well surface and then binding the antibody from serum. After binding and appropriate washing, the bound immune complexes are detected. Where the initial antigen specific antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first antigen specific antibody, with the second antibody being linked to a detectable label. This is the form of the assay which can have very high background when performed in the presence of high concentrations of serum.

Competition ELISAs are also possible in which test samples compete for binding with known amounts of labeled antigens or antibodies. The amount of reactive species in the unknown sample is determined by mixing the sample with the known labeled species before or during incubation with coated wells. The presence of reactive species in the sample acts to

reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal. Antigen or antibodies may also be linked to a solid support, such as in the form of beads, dipstick, membrane or column matrix, and the sample to be analyzed applied to the immobilized antigen or antibody. The current invention is distinct from competitive ELISA because the analyte is not measured on the basis of competition of unlabeled and labeled antigen for a limited number of binding sites. The displacement of the intact complex using hapten elution from the anti-hapten absorbent is competitive displacement, however.

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface. This approach is very useful, but does not completely prevent non-specific binding of analytes. This non-specific binding is increasingly noticeable when high concentrations of detector labeled antibodies are used or when highly sensitive assays are employed.

In standard ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of the antigen or antibody to the well, coating with a nonreactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand. It is not the custom in standard ELISA protocols to specifically elute bound complexes for subsequent analysis, as in the current invention, and in fact the inventor is not aware of any previous case of application of this principle.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background. The suitable

conditions also may mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 37°C, or may be overnight at about 4°C or so. It is important to recognize that, in the application of the current invention, it is essential to choose conditions for binding and assay which do not disrupt or destroy the complex analyte that one wishes to subsequently separate and further analyze.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove noncomplexed material. Washing often includes washing with a solution of PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of immune complexes labeled with detector may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, typical practice is to contact and incubate the first or second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation, *e.g.*, incubation for 2 hours at room temperature in a PBS -containing solution such as PBSTween. It is to be recognized that these enzymes are typically measured by using pH change (urease), oxidative reactions (glucose oxidase, peroxidase), or measurement at elevated pH (alkaline phosphatase). All of these detection methods are therefore likely to destroy the intact complex that one would wish to subsequently isolate.

It is therefore a requirement of the current invention that, when one wishes to separate and analyze further a complex analyte, it is necessary to employ a detection method which is non-destructive of the complex formed. In the inventor's laboratory, the method devised for sensitive measurement of analytes is the Enzyme-linked coagulation assay, or ELCA (U.S. patent # 4,668,621), which uses the coagulation cascade combined with the labeling enzyme RVV-XA as a universal detection system. The advantage of this system for the current invention, is that the coagulation reactions can be performed at physiological pH in the presence of a wide variety of buffers. It is therefore possible to retain the integrity of complex analytes. The present invention does not depend exclusively on the use of the ELCA method; alternative reactions for detection of bound analyte can be performed under gentle conditions using other detector

molecules. Examples applicable in selected cases include chemiluminescent labels, described in U.S. Patent Nos. 5,310,687, 5,238,808 and 5,221,605.

ELISA-ELCA

The inventor has previously developed and described an amplified immunoassay for botulinum toxin which matched the sensitivity of the mouse bioassay (Doellgast *et al.*, 1993; Doellgast *et al.*, 1994a; Roman *et al.*, 1994; Doellgast *et al.*, 1995). This assay is an enzyme-linked coagulation assay (ELCA). One modification of this assay depends on the formation of a complex in solution phase between the neurotoxin, enzyme-labeled antibody and hapten-labeled antibody. This assay uses a coagulation activator (Russell's viper venom factor X activator) as a labeling enzyme and a coagulation based assay for the detection of bound complexes.

Briefly, in ELISA-ELCA system, the two labeled antibodies (an RVV-XA-labeled antibody and a second labeled antibody, Abu) are incubated with a toxin containing sample for a period sufficient to allow complex formation. The time for complex formation can be varied according to the protocol being employed. In some instances the mixture is allowed to incubate from 0 to 120 minutes at 37°C or 1 to 2 days at 4°C. Once complex formation has occurred the complex is captured onto a solid support comprising an appropriate adsorbent that recognizes Abu. The reaction mixture is further incubated for an appropriate period of time. After the incubation period the plate is washed in a suitable solution containing saline.

Following the washing of the immunoabsorbed complex an appropriate ELCA substrate mixture is applied and the reaction mixture is again incubated at 37°C for an appropriate period of time. This incubation period is followed by incubation with alkaline-phosphatase linked fibrinogen in 1% triton X-100 added to the reaction mixture. The substrate for the RVV-XA antibody is factor X, which is hydrolyzed to its active form Xa, which complexes with factor V and hydrolyzes factor II to its active form IIa (also known as thrombin), which then can hydrolyze enzyme-labeled fibrinogen in solution and solid-phase fibrinogen added in the form of fibrinogen-coated plastic 'pegs'. The sequence of reactions occurring as part of the coagulation cascade initiated by RVV-XA (X→Xa, II→IIa, Fibrinogen→Fibrin) results in a very sensitive detection of the bound RVV-XA, reaching a lower practical limit of approximately 10^{-14} g RVV-XA/ml of solution. The plastic surface (polystyrene 'pegs' or silicone rubber 'nubs' have been used to date) are then placed in a second ELISA plate containing the alkaline phosphatase substrate solution comprising an appropriate colorimetric composition. This second ELISA plate

is incubated to allow the color from the colorimetric substrate to develop. Because the color generation from the bound alkaline phosphatase-fibrinogen occurs in a second plate, the plate to which the analyte is bound is not subjected to extreme conditions of pH and strong buffers, and consequently it is possible to retain the complex intact. The plastic pegs or nubs are removed from the now colored solutions in the wells of the second plate and the reaction is quantitated using standard plate reader techniques.

Sensitive Detection of Antibodies in Plasma

Following is a description of an amplified detection method for toxin specific antibodies that are present in low concentrations in the plasma, using non-denaturing conditions, according to the present invention. This is a detailed description of the application cited previously, appearing in Doellgast *et al.*, 1997.

An exemplary toxin antigen is the botulinum toxin. The antigen in this case is fairly large (molecular weight=140,000), and therefore has multiple binding sites (epitopes) for antibodies. If the complex was formed in the presence of human antibody, then the complex would preferably consist of three species of antibody, i.e., a hapten labeled (fluoresceinated) horse antibody, an RVV-XA-labeled horse antibody, and the human anti-toxin antibody. In the case of botulinum toxin, the three species may be, for example, fluoresceinated horse antibody (FlAb), RVV-XA-horse antibody (RVVAb) and human anti-toxin antibody (HuAb). The reaction would look like this:

$$\text{Fl-Ab} + \text{RVV-Ab} + \text{Hu-Ab} + \text{Toxin} \rightarrow \text{Fl-Ab*Toxin*RVV-Ab}$$
$$\text{*Hu-Ab}$$

where * indicates a binding complex.

The complex can then be bound to either antihuman antibody or antifluorescein antibody, preferably immobilized on a solid support, and in both cases the RVV-Ab bound to complex would be the basis for detection of the complex. If one attempted to bind the complex to anti-human antibody, then one must contend with the fact that the serum concentration of IgG is about 5-10 mg/ml. Since capture matrices have a limit of binding of 1-10 μg/ml of immunoglobulins, very little of the specific complex would be captured. However, if one captures the complex with antifluorescein, then the only human antibody species which will be specifically bound to the plate are those which are reactive with toxin or with horse

immunoglobulins. The capture of the complex by an anti-fluorescein antibody bound to a microtiter plate is depicted as:

Fl-Ab*Toxin*RVV-Ab + Microtiter plate anti-F1 (MP-AF) ⟶

MP-AF*Fl-Ab*Toxin*RVV-Ab
*Hu-Ab

Reactivity of components present in the sample (e.g. rheumatoid factor) with horse immunoglobulins can be prevented by adding excess unlabeled and unreactive horse IgG and tested using controls without added toxins. A 'specific' signal should not be apparent in assays in which toxin is omitted from the assay mixture. One can therefore be sure that the only human antibodies bound are those which are toxin-specific. The excess human immunoglobulins are washed from the plate, and the only human antibodies specifically bound to the plate *via* the antifluorescein capture antibody are those which are bound to the toxin complex. These complexes are specifically eluted in the presence of an excess of fluorescein, and are then transferred onto another matrix of antihuman immunoglobulin as follows:

MP-AF*Fl-Ab*Toxin*RVV-Ab --- 1 mM Fl ---> MP-AF*Fl + Fl-Ab*Toxin*RVV-Ab
*Hu-Ab *Hu-Ab

MP anti-IgG (MP-aIgG) + Fl-Ab*Toxin*RVV-Ab →Fl-Ab*Toxin*RVV-Ab
*Hu-Ab *Hu-Ab*MP-aIgG

The complex is bound to the capture plate only when the human antibody is present in the complex.

Of course it is understood that the botulinum toxin is only an exemplary toxin and virtually any toxin or other molecule present at low concentration will be detectable using this system. Any molecule may be employed as a label that offers the necessary sensitivity to detect low levels of antibody. Labels that may be used in the practice of the invention include, but are not limited to enzymes, radionuclides, fluorescers, chemiluminescers, enzyme substrates and co-factors, enzyme inhibitors and the like. The labels may be bound directly or indirectly to the antibody, there are numerous patents that describe the use of such materials, such as US. Pat. Nos. 3,654,090; 3,690,834; 3,817,837; 3,867,517; 3,935,074; 3,975,511; 3996345 and 4,020,151.

It is noteworthy that when the label is a hapten, it is not limited to fluorescein. Other exemplary haptens include, but are not limited to, rhodamine, phycoerythrin, phycocyanin,

allophycocyanin, texas red, and o-phthaldehyde, dansyl, dinitrophenyl, CY5 or any other detectable hapten known in the art. It is also recognized that the term hapten refers to a small molecule which can interact with an antibody; other ligand-receptor interactions of equivalent specificity, as for example the avidin-biotin system, could likewise be employed. Although the invention has been described as useful for the detection of a single antibody species in serum, it is contemplated that multiple assays may be performed on the same sample, then sorted out by binding to several different matrices and elution from these onto a single matrix. Alternatively, several assays can be bound to a single initial matrix and then bound subsequently to several distinct matrices. Thus, antibody to toxin could be captured on anti-fluorescein and then divided by reaction with anti-IgG, anti-IgM, anti-IgA and anti-IgD and/or anti-IgE. In another alternative, three different toxins could be assayed with a single mixture of specific antibodies reactive with all three toxins, with the capture antibodies being labeled with different haptens such as fluorescein, rhodamine and texas red, for example, and captured on appropriate antibody matrices, eluted and measured for IgG, IgA and IgM antibodies. In this way, the initial incubation to form complexes can allow multiple assays on a single sample which is fractionated and specifically measured by using specific hapten elution. In this case, nine separate analyses could be performed on a single serum sample containing anti-toxin antibodies.

It should also be stated that the second matrix could be a thin filter, thereby allowing the effective concentration of samples from crude mixtures, their elution and subsequent detection in very concentrated form. The effect of this is to eliminate very crude contamination problems and to develop highly sensitive assays. Starting with several milliliters of sample and ending in some cases with a filter having less than a microliter volume, trapping all the hapten eluted and bound complex.

The support to which the antibodies are captured can be present in a variety of forms. It may be conveniently bound to walls of microtiter wells, walls of capillaries, porous filters consisting of nitrocellulose, nylon or the like, bound to particles such as magnetic particles, polysaccharides, SEPHAROSE, agarose or the like, or any other surface which allows the complex to be localized at a site where the label may be measured. Of particular interest are microtiter plates from which the signal may be conveniently measured by use of commercially available microtiter plate readers.

ISOLATION OF BACTERIA

The present invention may further be employed to isolate bacteria. The inventor has been able to measure several bacteria specifically using surface antigens. These include the 0157 antigen of *E. coli* O157/H7 which produce shiga-like toxins and cause hemolytic uremic syndrome (HUS) and the 01 and 0139 antigens of *Vibrio cholerae* strains associated with pandemic outbreaks. This was accomplished using fluoresceinated and RVV-X-labeled antibodies and capture using anti-fluorescein, in a manner very similar to the case of the *C. botulinum* neurotoxin assay described in detail herein.

The assay of intact bacteria on microtiter plates is not regarded as a rigorous demonstration of assay specificity for bacteria. The bacteria must be viable and therefore isolated as colonies from the suspected source. The inventor also tested the growth of bacterial colonies in fluorescein and derivatives of fluorescein. It was found that, either in fluorescein or proteins derivatized with fluorescein (fluorescein-gelatin, fluorescein-casein), *E. coli* 0157/H7, *Vibrio cholerae* O1 and *Vibrio cholerae* O139 can grow without apparent inhibition under conditions which allow elution of analytes from solid-phase anti-fluorescein absorbents. It is therefore possible to elute bacteria from a solid phase on which they have been captured by anti-hapten - hapten labeled antibody as part of a specific assay using hapten for elution in growth medium and to then grow colonies for confirmation of the presence of viable bacteria.

DNA/RNA ASSAYS

A further embodiment of the present invention is to employ the methods described herein for the measurement of nucleic acids. As an example of the practice of this method, a dual-labeled DNA may be produced during PCR amplification of gene sequences. As an example, fluoresceinated and biotinylated DNA may be produced during a PCR amplification by techniques well known in the art, or can be prepared by hybridization of probes containing the same ligands to DNA/RNA present in cell extracts. This labeled DNA can be detected at very low levels.

In order to optimize sensitivity in hybridization assays, relatively high concentrations of reactive probes are used. This means that if the indicator probes are present in excess, they will bind to the plastic surfaces in quantities adequate to interfere with the specificity of the assay. This problem is strictly analogous to the binding of human antibodies, and can be solved using hapten elution in a very similar way.

If a dual-labeled complex of fluoresceinated-biotinylated DNA/RNA is bound to anti-fluorescein, then the complex can be labeled with enzyme-labeled streptavidin. Elution of this complex from the anti-fluorescein using fluorescein will yield a complex of biotinylated-fluoresceinated DNA/RNA with RVV-X-streptavidin bound which can bind to a biotinylated protein present on a second solid phase. This is possible because only one of the binding sites of the enzyme-labeled streptavidin is bound to the DNA/RNA. The RVV-X is present on the second solid phase only if it was bound to the first solid phase, eluted using fluorescein and capable of binding to the biotinylated protein present on the second solid phase. The advantage in this case is analogous to the elimination of 'background' activity in immunoassay of antibodies, since biotinylated probe will only bind to the anti-fluorescein matrix and be eluted when it is hybridized to the target DNA/RNA, and RVV-XA-streptavidin will likewise bind to the anti-fluorescein matrix and be eluted with fluorescein only when it is attached to the biotinylated probe complexed with the target DNA/RNA

Alternatively, a DNA/RNA molecule could be hybridized with three probes, one of which was fluoresceinated, one of which was labeled with digoxigenin, and one of which was labeled with biotin. These three ligands are commonly used in current probes. In this case, the hybridized molecule could be bound to anti-fluorescein, the bound molecule could be labeled with RVV-XA-streptavidin or some other streptavidin-indicator molecule, and then eluted with fluorescein or fluorescein-protein and bound to an anti-digoxigenin matrix. This approach is of particular value when the presence of three or more sequences on the same target molecule is indicative of the presence of the gene of interest. For example, the presence of the *inv*A-*inv*E genes adjacent to one another is a particular characteristic of *Salmonella* species (Stone *et al.*, 1994) If one uses one biotinylated probe having part of the *inv*A sequence, one fluoresceinated probe having part of the *inv*E sequence, and one digoxigenin probe reactive with an internal sequence at the juncture of the two genes, then the mixture of all three bound to the same DNA molecule would be apparent using the transfer elution approach described in this invention. This application is analogous to the identification of bacterial strains by comparison of their surface antigens as outlined above. It demonstrates the broad applicability of the current invention in achieving additional specificity for identification and characterization of any analyte with low background by use of the hapten elution approach.

The additional specificity indices indicated above are superior to the limited specificity of binding to the anti-fluorescein matrix, which is compromised by the possible 'non-specific'

binding of biotinylated DNA from the hybridization mixture or of RVV-XA-streptavidin. It is further to be noted that when binding to biotinylated fibrinogen is used to identify the RVV-XA-streptavidin eluted in the previous examples, the eluted complex is then bound to a protein which can be used to perform a coagulation assay after capture, thereby simplifying the protocol.

Dual-Labeled Capture Antibodies.

In many cases, it is desirable to use very high concentrations of labeled antibodies in immunoassays that aim at higher sensitivity. When, for example, a polyclonal antibody is used to detect an antigen, the polyclonal antisera will consist of multiple antibody populations which differ in affinity. In order to have the highest affinity antibodies represented in the labeled conjugate, one would typically use very high concentrations of labeled antibodies and try to minimize non-specific binding. Also, since affinity chromatography (i.e. purification of antibodies on absorbents with bound antigen) may not allow one to recover the highest affinity antibodies under conditions which do not degrade the bound antigen, it is sometimes necessary to use antibodies which have not been affinity-purified and so contain a high 'background' of immunoglobulins not specifically reactive with the analyte. When these are labeled with detector ligand, the problem of non-specific binding can significantly decrease the sensitivity of the assay. The hapten elution technique which is central to the current invention can be very effective in these cases in reducing non-specific binding in the final analysis. If an antibody is labeled with both biotin and fluorescein, or if a chicken or mouse (e.g., monoclonal) antibody is labeled with fluorescein, then complexes can be captured onto anti-fluorescein, eluted and bound to streptavidin, anti-chicken or anti-mouse IgG in order to get the optimal specificity for formation and detection of complex without the additional background resulting from the binding of the enzyme-labeled antibody added in excess to the initial anti-fluorescein matrix. If the detector-labeled antibody is bound non-specifically, it will not be eluted specifically by fluorescein, and so the amount of conjugate bound to the second matrix will reflect the amount of analyte without detectable background. In such an application, one would typically not measure the amount of analyte bound to the anti-fluorescein matrix, since this would be used only as an initial 'trapping' matrix to allow specific binding to the second matrix with no or much decreased 'background' activity. It should be noted that porous filters, which have very high 'background' binding as a result of their extremely high surface area, would be of particular use in this application of the invention, since they are convenient devices to effect transfer from one surface

to another, and when a sample is concentrated on a filter there can be substantial reductions in volume which can further enhance the sensitivity of an analysis.

Antibody Production

Means for preparing and characterizing antibodies are well known in the art (See, e.g., Harlow and Lane Eds., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

Antibodies against any antigen of choice will be useful in the practice of the present invention. These antibodies will generally be of one type and will be used to bind to the toxins or other antigens to be investigated so that they can be used in assay for the detection of such antigens and for the detection of antibodies to such antigens using the methods of the present invention.

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig, a goat, a horse or a chicken.

Immunogenic compositions comprising toxins such as botulinum toxins, anthrax, Diphtheria, Tetanus, Shiga toxin (ST) and Shiga-like toxins (SLT) produced by some bacterial strains, Cholera toxin (CT) produced by species of *Vibrio cholerae*, the related heat-labile enterotoxin (LT) produced by *E. coli* strains, Staph enterotoxins, and other toxins which induce pathological reactions in humans or animals, fragments of these toxins and the like. The immunogenic compositions detected by the present invention may also be useful in veterinarian medicine. As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a compound to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimideester, carbodiimyde and bis-biazotized benzidine. In the case of toxins, chemical treatment to enhance immunogenicity also includes treatment to reduce toxigenicity, especially in the case of neurotoxic proteins such as botulinum and tetanus toxins.

The immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant. For toxins, distinct from most antigens, it is generally necessary to inhibit their biological activity before they can serve as antigens by preparing chemically altered forms known as 'toxoids'. For botulinum toxin, this is traditionally accomplished by formalin treatment of the toxin.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the

spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 6566, 1986; Campbell, pp. 7583, 1984). cites). For example, where the immunized animal is a mouse, one may use P3X63/Ag8, X63Ag8.653, NS1/1.Ag 4 1, Sp210Ag14, FO, NSO/U, MPC11, MPC11X45GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3Ag 1.2.3, IR983F and 4B210; and U266, GM1500GRG2, LICRLONHMy2 and UC7296 are all useful in connection with human cell fusions.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the medium is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid. Radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like, may be used.

The selection method used for identification of appropriate monoclonal antibodies in the inventor's laboratory can include an estimation of the relative affinity of interaction. This can be accomplished by binding the antibody from culture fluid to a test plate consisting of anti-mouse IgG, then adding various concentrations of antigen labeled with RVV-XA. High affinity antibodies will bind to low concentrations of labeled antigen, and will not dissociate after subsequent washing and re-assay of bound labeled antigen. This approach was used to identify high and low affinity monoclonal antibodies to RVV-XA for example.

Where one desires to generate an antibody with defined activity, one would generally screen the candidate hybridomas to identify those hybridomas that produce antibodies that have the desired inhibitory or stimulatory properties.

Any selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if

desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

Enzyme Conjugates

Intrinsic to the ELISA and other ELISA based assays is the addition of reagents conjugated to enzymes. Assays are then quantified by the build-up of colored product after the addition of substrate or substrate and dye combination. Usually antibodies are conjugated to enzymes. Other commonly used systems involve the conjugation of enzymes to pseudo-immune reactors, such as protein A and protein G (which binds to mammalian IgGs), and indirect labeling using biotin-avidin systems. Tables 1 and 2 show properties of enzymes, substrates, and stopping conditions.

Horseradish Peroxidase (HRPO) Plus Hydrogen Peroxide Substrate

The horseradish peroxidase (HRPO)/ hydrogen peroxide substrate is widely a used enzyme system in ELISA type reactions. The reduction of peroxide by the enzyme is achieved by hydrogen donors that can be measured after oxidation as a color change. The choice of converted substrates that remain soluble is essential in ELISA, so that optimal spectrophotometric reading can be made. Commonly used chemicals are O-phenylene diamine (OPD), 2,2'-aznodi-ethylbenzothiazolinesulfonic acid (ABTS), 5-aminosalicylic acid or tetra-methylbenzidine (TMB), all of which are commercially available.

Table 1

Enzyme/Substrate Systems for ELISA

Enzyme label (mol wt)	Substrate	Dye	Buffer
Horseradish peroxidase (40,000)	H_2O_2 (0.004%)	Ortho-phenylene diamine (OPD)	Phosphate/citrate (0.1M), pH 5.0
	H_2O_2 (0.004%)	Tetra-methylbenzidine (TMB)	Acetate buffer (0.1M), pH 5.6
	H_2O_2 (0.002%)	2,2'-Azinodi-ethylbenzothiazoline-sulfonic acid (ABTS)	Phosphate/citrate (0.1M), pH 4.2
	H_2O_2 (0.006%)	5-Aminosalicylic acid (5AS)	Phosphate (0.2M), pH 6.8
	H_2O_2 (0.02%)	Di-aminobenzidine (DAB)	Tris or PBS, pH 7.4
Alkaline phosphatase (100,000)	pnpp (2.5 mM)	Para nitrophenyl phosphate (pnpp)	Diethanolamine (10 %) and $MgCl_2$ (0.5 mM), pH 9.5
	Phenolphthalein monophosphate (5 mM)	Phenolphthalein	2-amino-2-methyl-1,3 propanediol (1 M) and $MgCl_2$ (5 mM), pH 9.8
b-galactosidase (540,000)	ONPG (3 mM)	O-Nitrophenyl b-D-galactopyranoside (ONPG)	$MgCl_2$ and 2ME (0.01M)/PBS, pH 7.5
RVV-XA	Factors II-V-X-lipid	Enzyme labeled fibrinogen and solid-phase fibrinogen. Any of the other enzymes in this list may be used to label fibrinogen, and bound enzyme detection can be accomplished	Gelatin-containing buffer, pH 7.5-8 with 3 mM $CaCl_2$. Conditions can be varied widely with respect to salt concentration and buffer composition.
Urease (483,000)	Urea	Bromocresol	pH 4.8

Table 2

Enzyme/Substrate Systems for ELISA(2)

Enzyme/Substrate Systems for ELISA(2)

Enzyme label	System	Color change		Reading wavelength, nm		Stopping solution	Notes
		Nonstopped	Stopped	Nonstopped	Stopped		
Horseradish peroxidase	OPD	Green/ orange	Orange/ brown	450	492	1.25*M* H_2SO_4	Possibly carcinogen, soluble product
	TMB	Blue	Yellow	650	450	SDS (1%)	Nonmutagenic, soluble product
	ABTS	Green	Green	414	414	No stop	Mutagenic, soluble product
	5AS	Brown	Brown	450	450	No stop	Safe, soluble product
	DAB	Brown	Brown	N/A	N/A	No stop	Insoluble product, safe
Alkaline phosphatase	Phenolph P	Red	Red	550	550	No stop	Safe, soluble product
Alkaline phosphatase	pnpp	Yellow/ green	Yellow/ green	405	405	2*M* sodium carbonate	Safe, soluble product
β-galactosidase	ONPG	Yellow	Yellow	420	420	2*M* sodium carbonate	Safe, soluble product
Urease	Urea bromocresol	Purple	Purple	588	588	1% merthiolate	Safe, soluble product

The optimum substrate (hydrogen peroxide) concentration depends on the hydrogen donor and the solid-phase. This is usually established in preliminary tests, but concentrations between 0.010 and 0.0005% are adequate. Hydrogen peroxide is available as 30% commercially. The development of the colored product is measured at different wavelengths. The optimum wavelength may also shift if the reaction is stopped by a blocking reagent to prevent change in the optical density after a reaction period.

The stopping reagents involving HRPO are: solutions of hydrochloric or sulfuric acid for OPD and TMB; and sodium dodecyl sulfate (SDS) for ABTS.

The optimal wavelengths for reading are:

415 nm for ABTS;

492 nm for acidified OPD (420 nm for nonacidified OPD);

492 nm for 5-AS; and

655 nm for TMB (unstopped) and 450 nm (acidified).

Alkaline Phosphatase Plus p-Nitrophenylphosphate

Another commonly used enzyme system alkaline phosphatase/p-nitrophenylphosphate which is assayed in buffer depending on the source of the enzyme. For bacterial enzyme, 0.1*M* Tris-HC1 buffer, pH 8.1, containing 0.01% magnesium chloride is used. For intestinal mucosal enzyme, a 10% (w/w) diethanolamine (97 mL in 1 L of a 0.01% magnesium chloride solution) buffer, pH 9.8 (adjusted with HC1), is used. The *p*-nitrophenylphosphate is added just before use (available as preweighed pellets) to 1 mg/mL. The production of nitrophenol is measured at 405 nm. The reaction is stopped by the addition of 0.1 vol of 2*M* sodium carbonate. Note that inorganic phosphate has a strong inhibitory effect on alkaline phosphatase, and therefore, PBS or similar buffers are avoided.

Alkaline Phosphatase plus phenolphthalein monophosphate.

This system has principally been used in the development of the ELISA-ELCA system. In this case, advantages of this substrate include the fact that the stock solutions of the phenolphthalein monophosphate are stable for months at room temperature, and the product of the reaction is a bright red dye readily visible to the naked eye. Otherwise, the performance of the assay for alkaline phosphatase using this substrate is identical to that for paranitrophenyl phosphate. Because the inventor uses this assay for measurement of deposits of alkaline phosphatase-labeled fibrin onto solid phases, the inventor does not 'stop' the reaction. It is therefore possible to wash off the solid phases containing bound enzyme-labeled fibrin and to

add them again to the substrate mixtures containing thrombin and alkaline phosphatase-fibrinogen to get further deposition of the labeled fibrin for added sensitivity.

β-Galactosidase Plus O-Nitrophenyl-β-Galactopyranoside

The β-galactosidase system is determined after addition of a solution containing 70 mg *o*-nitrophenyl β-D-galactopyranoside/100 mL of 0.1*M* potassium phosphate-buffer, pH 7.0, containing 1 m*M* magnesium chloride and 0.01*M* 2-mercaptoethanol. The reaction may be stopped by the addition of 0.25 vol of 2*M* sodium carbonate.

ELCA assays for detection of analytes.

The ELCA assay system is a comprehensive system for detection of the process of coagulation, which is the polymerization of fibrin. Used as an amplification system, it is therefore possible to use any clotting factor or protease which can induce clotting from any source and get the same end result, i.e. the polymerization of fibrin. In the current practice, the enzyme from a snake native to Southeast Asia, the Russell's viper, produces as 5-8% of its venom an enzyme known as Russell's viper venom factor X activator, or as the inventor has defined it RVV-XA (generally known by the abbreviation RVV-X). The advantages of this enzyme are its high concentration (which makes it relatively easy to purify) and the fact that it induces coagulation relatively early in the coagulation cascade, and can therefore be measured with high sensitivity (less than 10^{-14} grams per milliliter, or 1 part in 100 trillion). However, it is apparent that any other enzyme or cofactor which can activate coagulation could be employed in the assay.

This capability of the assay can be employed to provide additional analytical flexibility for the current invention. For example, one may elect to use RVV-XA as a label to indicate the presence of the original analyte captured onto anti-hapten, and then after capture at a second stage of the assay use the label factor Xa conjugated to a second antibody or probe. The substrate for this would be a mixture of factors II, V, lipid, enzyme labeled-fibrinogen and solid-phase fibrinogen. This substrate would not detect the original RVV-XA, since it does not contain factor X but would detect the activated factor X (i.e. Xa). Similarly, if factor Va were used for labeling, then a substrate consisting of Xa, II, lipid and enzyme-labeled fibrinogen would detect the bound Va. It was also noted in the study of the use of this assay system for avian clotting factors (Gupta *et al.*, 1993) that avian (pigeon, duck) Xa does not interact with mammalian Va, so that the same process could be repeated with purified avian clotting factors in the presence of bound mammalian clotting factors (RVV-XA is apparently equally effective in

activating coagulation in both avian and mammalian species). Finally, additional enzymes obtainable from other species could be employed; for example the factor II activator obtainable from Taipan Snake Venom (also known as TSV-II) requires only the substrate II + lipid + labeled fibrinogen + solid phase fibrinogen for measurement. In the presence of any or all of the above analytes (RVV-XA, Xa, Va) bound to the solid phase, TSV-II would be uniquely detectable because it alone would have activity in the absence of the factors needed for earlier steps in the coagulation cascade. At each stage of the hapten transfer assay using the ELCA system for detection the previous substrate mixture is washed away with buffer and a new mixture of clotting factors including solid phase fibrinogen is added to the bound analyte. This ability to search for additional specificities using not only different capture ligands but also different detector ligands is therefore a powerful adjunct.

Another valuable feature of the ELCA system for the current application, is that the nature of the label is not important for the detection process. It has been possible to label fibrinogen with a wide variety of labels including all enzymes, fluorescein, rhodamine, biotin, gold beads etc. All of these derivatives are able to polymerize into fibrin. Thus, any detection system including all of the enzymes listed above, solid phase systems organized onto 'biosensors' and systems using labeled microbeads including gold beads and various colored beads can be employed to detect complexes. Since many molecules of fibrin are deposited for each molecule of labeled analyte bound to the anti-hapten absorbent, the sensitivity of these various techniques are all increased by using the ELCA system for detection. Also, the conditions established with one label, as for example using microtiter plates to determine optimal analyte concentrations, are applicable to the new detection system by simply replacing the solid phase and labeled fibrinogen source. An example would be the evanescent wave biosensor, which has also been applied for the measurement of *C. botulinum* neurotoxin at much lower sensitivity than the ELISA-ELCA technique (i.e. much higher concentrations of toxin are needed for the present embodiment of this technique to obtain measurable signal than for the ELISA-ELCA assay). The problem in this case, is accomplishing the binding of enough analyte to get a detectable signal using this rapid detection device. If instead of attempting to get fluorescent antibody to bind with adequate efficiency to the tapered optical fiber which is integral to the device, one were to bind labeled fibrin, the desired sensitivity could easily be reached. Any system for deposition of labeled fibrin onto appropriate solid phases as an endpoint of the ELCA

system to take advantage of greater convenience/speed of analysis are envisioned in the general use of this system for detection of labeled complexes in the current invention.

Urease, pH Change, and Bromocresol Purple Indicator

The urease enzymes system is determined by addition of a weakly buffered solution of urea (pH 4.8) in presence of bromocresol purple. The urea is hydrolyzed to liberate ammonia in the presence of urease, and this raises the pH of the solution resulting in a color change from yellow to purple.

The reaction can be stopped by the addition of 10 μL of a 1% solution of merthiolate (thiomersal) to each well. Tables 1 and 2 summarize properties of various enzyme systems.

Conjugation With Enzymes

The sensitivity of the ELISA depends on the ability of the antibody to bind and the specific enzyme activity of the labeled immunoreactant, the conjugate. The linkage of an enzyme to an antigen or antibody may affect the specificity of an assay if any chemical modification of the moieties involved alters the antigenic determinants or the reactive sites on antibody molecules. Thus, chemical methods that do not affect these parameters have been chosen. Not only the immunoreactivities, but also the catalytic activity of the enzyme must be maintained after conjugation. Following conjugation, it is necessary to test the immunoreactivity as to whether it has the desired specification. Before use in ELISA, it may be necessary to purify the conjugates to remove unconjugated antigen or antibody and free enzyme. Reagents used to produce conjugates are numerous, and their mode of action is to modify the functional groups present on proteins. Antigens that are non-proteinaceous, *e.g.*, steroids, can be conjugated with different means and are not dealt with here. Enzymes are covalently bound to reagents either directly by reactive groups on both enzyme and reagent or after introduction of reactive groups (*e.g.*, thiol or maleimid groups) indirectly *via* homo- or heterobifunctional reagents in two-step procedures. In the case of conjugation, the procedure which has proven most effective has been the conjugation using sulfhydryl derivatives of antibodies and antigens, coupled to RVV-XA using a bifunctional reagent having both succinimidyl and maleimido entities (Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate). The bifunctional reagent is first reacted with the RVV-XA molecule, and then the reduced immunoglobulin is reacted with this molecule. This has proven to meet the requirements for optimal conjugation, which are:

1. Simplicity and rapidity;
2. Reproducibility (obtaining constant molar ratio of enzyme and reagent);
3. High yield of labeled reagent, and low yield of polymers of enzyme and reagent;
4. Low-grade inactivation of reagent and enzyme;
5. Simple procedures for separation of labeled and unlabeled reagents; and
6. Long-term stability without loss of immunological and enzymatic activities.

Conjugation with haptens.

In the case of conjugation with haptens, there is the advantage that haptens are generally very stable organic molecules, and can be obtained with a wide variety of derivatization possibilities. For the special case of derivatization of antibodies with two distinct labels as used in this invention, it is desirable to use two distinct reactivities for labeling and then to isolate the antibodies which incorporate both reactivities. For example, labeling of mammalian IgG molecules can readily be accomplished using reduction of the hinge region disulfide using dithiotreitol at pH 6.0, separating the reduced IgG at pH 7.8 by molecular sieving, and binding it to fluorescein maleimide. This cysteine-derivatized molecule can subsequently be derivatized with succinimidyl or isothiocyanate derivatives of other haptens which react predominantly with amino groups, or with hydrazide derivatives which would react with oxidized carbohydrates. Once labeled, the greatest efficiency of use of these derivatives would be by affinity-purification of the hapten-labeled immunoglobulins on anti-hapten absorbents. Thus, a dual-labeled antibody which bound to and was eluted from anti-fluorescein and anti-rhodamine, for example, would be an effective reagent for first binding to anti-fluorescein and then to anti-rhodamine absorbents for the hapten elution approach of the current invention.

Development of Product of Enzymatic Reactions

The substrate for an ELISA type assay is usually chosen to yield a colorimetric product. The rate of color development will be proportional, over a certain range, to the amount of enzyme conjugate present. On a kinetic level, reactions are distinguished by their kinetic order, which specifies the dependence of reaction rate on the concentration of reactants. Under the conditions generally employed in ELISA, the reaction exhibits zero order with respect to the substrate. It can be seen that too little substrate will limit the rate of product production. Thus, sufficient substrate must be present to prevent the substrate and/or cofactors from being rate-limiting. Where substrate and chromogenic hydrogen donors are necessary for color development, the concentrations of both must be assessed to obtain optimum conditions. The

product must be stable within a defined time, and products that are unstable in bright light or at temperatures at which the assay is performed should be avoided. The physiochemical parameters that affect the development of color include:

1. Buffer composition and pH;
2. Reaction temperature;
3. Substrate and/or cofactor concentration and stability;
4. Product stability;
5. Enzyme stability; and
6. Substrate and product stability.

Horseradish peroxidase is active over a broad pH range with respect to its substrate, hydrogen peroxide. However, the optimum pH for the development of label in the ELISA will vary depending on the chromogenic donor. Changing the pH will reduce the reaction rate, but will not affect the reaction kinetics, *e.g.*, increasing the pH to 5.0 for ABTS will slow down the rate of reaction (pH optimum 4.0), but does not affect the linearity of the kinetics. The majority of the buffers used in substrate formulation are of low molarity citrate base. Since the reaction kinetics are dependent on pH, a stable buffering capacity is essential. The stability of HRPO varies in different buffers, being more stable in 0.1*M* citrate than 0.1*M* phosphate-buffers. High molarity phosphate buffer can be particularly damaging to HRPO at low-pH. Nonionic detergents exert a stabilizing effect on the enzymic activity of HRPO, and this can be enhanced by increasing reaction temperatures. The detergents have also been demonstrated as having a stabilizing effect on the enzymes.

Alkaline phosphatase is active at alkaline pH and optimum above pH 8.0. The buffers used with the substrate pnpp are diethanolamine/HCL, pH 9.6. Inorganic Mg^{2+} is essential for enzymic activation. Nonionic detergents appear to have no effect on the enzyme activation, substrate catalysis, or product development. Inactivation of the enzyme on contact with microplates does not occur.

Urease is enzymatically active over a broad pH range. The specificity of urease for its substrate (urea) is almost absolute. The urease substrate solution contains urea and a pH indicator, bromocresol purple, at pH 4.7. The urease catalyzes the urea into ammonia and bicarbonate. The released ammonia causes an increased in pH, which changes the color of the indicator from yellow to purple. The generation of color is not directly related to the amount of

urea catalyzed. Since the color development is dependent on pH, it is essential to check that the pH is accurate before addition. It is also essential that no alkaline buffers remain after, for example, washing (pH 7.4, PBS), since this will cause a change in color, and plates must be washed finally in water if PBS is the usual washing buffer.

The ELCA assay system depends on having conditions for stabilization of substrates including lipid and clotting factors. Conditions must be used to be certain that all of the components of the reaction can be stored under the appropriate conditions, and that the activatable (proenzyme) clotting factors remain in an inactive form during storage. It is also necessary to establish optimal buffer conditions for activation by the labeling enzyme and for polymerization of fibrin. It is also necessary for endpoint readings on the coagulation cascade to stop the reaction by addition of detergent for example (reactions depend on the presence of a lipid micelle) or by removing the surface containing the bound conjugate from the ELCA reaction mixture. It is also possible in this case to add a single clotting factor (factor X, for example) or a mixture of clotting factors (X, II, V) without the presence of fibrinogen and generate active enzyme which is subsequently transferred to the detector system which would include solid-phase and detector ligand-labeled fibrinogen. This flexibility permits the design of various formats depending on the desired sensitivity. Factor X is stable in solution, but mixtures of X, II and V always develop 'background' activity when mixed together. This background is not too high for the assays developed to date, but if additional sensitivity is desired it may be desirable to incubate factor X with RVV-XA for longer periods of time to get as much Xa as possible, then measure the Xa generated using mixtures of II, V, lipid, labeled fibrinogen.

Reaction Temperature

Between-well variation in an assay can cause differential rates of color development. Similarly, varying temperatures in the performance of the assay can cause variation. It is advisable, therefore, that substrates be added at a defined temperature and that plates be incubated under uniform conditions. This is normally room temperature. A preferred practice is to add substrate solutions at a defined temperature obtained by using solutions heated (or cooled) to that defined temperature. This is particularly important when attempting to standardize assays between operators and laboratories where a fixed time for stopping an assay is used. In the present use of the ELCA system, the inventor has adopted the custom of 'floating' microtiter plates in which the ELCA assay is performed in water baths set at 37°C. This brings the bottom

surface of the plate to a uniform temperature very quickly and maintains the plates in a moist environment.

Substrate/Cofactor Concentration and Stability

As already stated, optimization of substrate concentrations must be made. This is usually stated for particular systems (literature, kits, and so on). Certain solutions can be made and stored. As an example, OPD can be made up in buffer and stored frozen in well-sealed vials. It can then be thawed and used (after the addition of H_2O_2). This negates the need to weigh out small amounts of OPD for small volumes of substrate solution and aids standardization of assays. The use of preweighed chemicals in the form of tablets available commercially also greatly improves the accuracy and convenience of producing substrate solutions, although these tablets are expensive. For the mixtures of clotting factors used in the ELCA system, the concentrations are typically very much lower than those present in normal plasma. This is true because enough time must elapse to allow solid-phase polymerization of dilute enzyme-labeled fibrin. It is possible to vary the concentration of clotting factor substrate over a very wide range for selected applications, depending on the speed and sensitivity of analysis desired. Specific coagulation reactions in whole blood, for example, occur in 10-100 seconds, while the ELCA system uses 10-50 minutes at a concentration of clotting factors equivalent to 100-1000 fold diluted plasma. Any range between these two extremes could be reached with the appropriate mixture of clotting factors.

Product Stability

Once the substrate has been catalyzed and a colored or, in the case of labeled fibrin, solid-phase polymerized product achieved, it is essential that the color remains stable. In the majority of ELISAs, positive results are read by eye or by spectrophotometer, since the intensity of color (optical density) is compared to a series of previously worked out negative values. In the case of the ELCA system, the enzyme-labeled, polymerized fibrin bound to the solid phase can be remains active unless subjected to denaturing conditions or conditions which inactivate the bound enzyme (EDTA for alkaline phosphatase, for example). This permits one to keep the 'pegs' with bound alkaline phosphatase-fibrin in the stable phenolphthalein monophosphate substrate for any desired period of time to allow further color development.

An unstable colored product would affect the build-up of color. For spectrophotometric reading of results, it is vital that the product color remains stable without shifting the absorption

spectrum, since the microplate readers assess the absorbance of the colored product at a preset wavelength. Generally, enzymic activity is prevented from proceeding further at a predetermined time by the addition of a reagent, preventing further enzymic activity. Alternatively, for the ELCA system, removal of the labeled 'pegs' from phenolphthalein monophosphate in the current format stops the development of color.

Substrate and Product Stability

As already indicated, where substrates are only soluble to a limited extent in aqueous buffers, the use of mixed aqueous/organic buffers is possible. These solvent systems can allow significantly greater amounts of substrates to be incorporated into solution and allow their use in microplate ELISAs. Partially or totally insoluble products have their uses in variants of ELISA, *e.g.*, in the staining of sections in immunohistochemistry where insoluble products localize the area of antigen or antibody reaction. For the ELCA system, all substrates are soluble in solution.

Stopping Reactions

Reagents are added to prevent further enzymic reaction in ELISA and ELCA reactions. This is performed at a time as determined in the specific assay. This process is usually called "stopping," and the reagent that is used the "stopping reagent." The stopping is usually made at a time when the relationship among the enzyme-substrate-product is in the linear phase. Molar concentrations of strong acids or strong bases stop enzymic activity by quickly denaturing enzymes. Other stopping reagents are enzyme-specific. For example, detergent stops the generation of thrombin in the ELCA system but does not inhibit thrombin, and removal of enzyme-labeled fibrinogen coated 'pegs' from substrates stops the generation of color.

Sodium azide is a potent inhibitor of HRPO, whereas EDTA inhibits alkaline phosphatase by the chelation of metal ion cofactors. Since addition of stopping agents may alter the absorption spectrum of the product, the absorption peak must be known.

Thus, *e.g.*, sulfuric acid-stopped OPD/ELISAs are read at 492 nm (450 nm before stop). The addition of stopping agents can also increase the sensitivity of an ELISA. In the addition of stopping reagent, the volumes must be kept accurate, since photometric readings are affected if the total volume of reactants varies.

Reading

Since the product of substrate catalysis is colored, it can be read in two ways, namely, (1) by-eye inspection or (2) using spectrophotometers.

By-Eye Reading

ELISAs can be designed for use with either system, although different conditions and controls may have to be included. It is essential that the principles of ELISA be thoroughly understood before either system is adopted. In particular, the by-eye test is not necessarily simpler to standardize. However, where correct standardization is used, it offers sensitive assays. When a correct plate template is used, the range of color product will be from full color through partial color to no color.

Known strong positive samples will give strong color. Weak positives will give partial color, and negatives will give no color, or that of negative wells. It is essential that controls of this sort be incorporated in the intended assays. Some difficulties arise in differentiating weak positives from negatives by-eye. The interpretation of by-eye tests can vary from operator to operator, and hence, results are more subjective than by spectrophotometer. Some substrate/enzyme combinations favor by-eye reading. This is particularly true of phenolphthalein monophosphate, which is yellow in color, compared with phenolphthalein, which is red.

Where tests have to be read by-eye (where instrumentation is not available), the best assays can be produced in other laboratories that can quantify reagents using machine reading and evaluate the parameters of the by-eye reading. As an example, a negative population of sera can be examined, and control negative sera, reflecting different parts of the negative OD distribution, can be adopted for by-eye controls. Thus, a serum having the highest OD value may be selected as the negative control. Any sera giving by-eye discernible results higher than this serum would therefore be assessed with high confidence as being positive. Assays that require comparison of closely related data, such as competition assays, are not suitable for by-eye interpretation, *e.g.*, where the competition slope is compared.

Spectrophotometric Reading

The product of the substrate catalysis by enzyme is measured by transmitting light of a specific wavelength through the product and measuring the amount of adsorption of that light, if any, by a machine. Since different products are produced in ELISA, care is taken to select appropriate filters for the detection of the correct wavelengths. Although microcuvets and conventional spectrophotometers can be used for this purpose, this is laborious where large numbers of samples are measured. Special machines are available for the reading of colored products in microplates. These read the absorbance of each well at a preselected wavelength of

light. Either one well can be read at a time (manual readers) or more suitably, a column of eight wells is read simultaneously (semiautomatic or automatic multichannel spectrophotometers).

For the semiautomatic readers, the wavelength filters are added manually, whereas for the automatic readers, the wavelength filter(s) (dual-wavelength machines are available) can be selected from a control panel. In the main the basic results from such a machine are expressed as absorbance units and are recorded on paper rolls. Various (limited, but useful) processing of the data is usually available, such as the expression of the absorbance values as a matrix or as + and - against control wells or values given to the machine. Most readers can be connected to computers, and a range of software (commercial and private) is available to manipulate and store data. This is important in large-scale sample handling or where complicated arithmetic routines are performed on the data. An important feature of the ELISA having a colored product that can be examined by-eye is that tests can be rapidly assessed before machine reading. Thus, one can see that a test has "worked" or not at a glance. Extensive reading time is not wasted if a silly mistake has occurred, unlike RIA, where it is essential to count samples before results are obtained. Such by-eye assessment is also convenient when "sighting" experiments are being made during development of assays.

Kits

An aspect of the present invention is a kit for the detection of various antibodies and antigens. The kits of the present invention are designed to contain the reagents necessary for a particular detection, or they may be designed for a more generic utility. As examples, described below are the most completely defined applications, the detection of antibodies to *C. botulinum* neurotoxin and the same toxins, and then give various examples of kit compositions which could use the same principle for a variety of analytes.

Human antibodies to <u>C. botulinum</u> neurotoxins.

The composition of this kit would include the following components:

1. A mixture of fluoresceinated and RVV-XA-labeled horse antibodies reactive with any of the toxins (e.g. toxin A, B or E). This could be provided in bulk form or in individual tubes in amounts adequate for a single sample.
2. A preparation of toxin at concentrations adequate for the analysis. This could also be provided in bulk form or in individual tubes. For the assays used to date, a final concentration of 10 ng/ml of toxins A or E, and 20 ng/ml of toxin B have proven optimal.

3. Microtiter plates coated with goat anti-fluorescein or monoclonal antibody anti-fluorescein.
4. Microtiter plates coated with anti-human IgG. For specific applications, coating with anti-IgM or anti-IgA may be substituted.
5. A solution of buffer containing fluoresceinated gelatin with a fluorescein concentration of 0.1 mM; autoclaved or sterile filtered.
6. ELCA substrate components including clotting factor mixtures, buffers, and fibrinogen-coated 'pegs' as well as a flat-bottom plate and alkaline phosphatase-fibrinogen.

The assay would be performed by dilution of the serum sample into buffer in the anti-fluorescein-coated microtiter plate, followed by addition of the labeled horse antibody mixture and finally the toxin. This could also be done in tubes containing the antibody mixture, which could then be added to tubes containing the appropriate dried toxins and transfer into the anti-fluorescein plate. Controls would be antibody dilutions added to labeled horse antibody mixtures but without added toxin. After incubation for several hours to several days at temperatures varying from 4°C to 37°C, the anti-fluorescein plate would be washed with saline and then soaked with detergent buffer for at least 10 minutes. The plate would then be treated with the fluorescein-gelatin solution for several hours to several days at temperatures varying from 4°C to 37°C. The complexes eluted from the plate would then be transferred to a second (anti-human IgG) plate and allowed to bind to this for several hours to several days at temperatures varying from 4°C to 37°C. The bound complex would then be assayed by ELCA.

Numerous variations of this kit are possible. For example, the anti-fluorescein plate could be replaced with an anti-fluorescein column or porous filter. This would increase the speed of binding of the complex once formed and simplify its elution and transfer. The eluate could be tested on plates of anti-IgG, anti-IgA and anti-IgM in order to assess the relative contribution of these three antibody types. This could be of special value in discerning the early (IgM) response to an antigen. Alternatively, the solid phase absorbent could contain all three antibody specificities so that all reactive antibodies would be simultaneously determined. Anti-light chain (kappa, lambda) and/or mixtures of antibodies to all three types could accomplish this.

Several antibody mixtures could be incorporated into the same mixture, with the 'capture' antibody labeled both with fluorescein and some other ligands, as for example rhodamine, texas red, and dansyl. The fluorescein eluate could then be bound to a series of filters consisting of anti-rhodamine, anti-texas red, anti-dansyl, and the complexes of these would then be eluted by

the appropriate ligand bound to gelatin, for example, and bound to anti-IgG or the other absorbents, as needed. Although these would seem to be tedious variations, most of them could be accomplished by design of various absorbents to accomplish the elution and selective binding of these components on filters with minimal manipulation.

Instead of a toxic antigen, the antigen could be modified so that it was in a biologically inactive but antigenically active form ('toxoid'). Similarly, the same method in general could be developed by selective labeling of such a toxoid with hapten and RVV-XA. Since the *C. botulinum* toxin molecule consists of two non-identical subunits, each antibody which has at least two antigen-binding sites will bind at random to combinations of fluoresceinated antigen and RVV-XA-antigen. In this case, the labeled toxoid mixture is mixed with diluted serum, and a complex is formed with the two forms which binds to and is eluted from the anti-fluorescein absorbent in a manner very similar to the binding of the labeled horse antibodies.

Modification of the assay for antigen (toxin) detection.

The same system used for antibody detection has significant potential application for measurement of antigen. For botulinum toxin, in particular, it is very difficult to work with pure antigen used for purification of antibody. The antigen is the most toxic protein known, and is consequently dangerous and simultaneously expensive to work with. It would be highly desirable if antibody which was not affinity purified was usable for the assay with little background. The approach of dual-labeling of the capture antibody with several ligands would work well for this problem. Whole serum immunoglobulin fractions from hyperimmunized animals could be labeled with two haptens, one of which was anti-fluorescein in every case, and the other was a toxin-specific hapten. The whole antiserum antibodies would also be labeled with RVV-XA, for example, and kits would contain mixtures of the RVV-XA and hapten labeled antibodies to all three antigens. When bound to the anti-fluorescein absorbent, all samples would appear positive if the amount of bound RVV-XA were determined because of the high concentrations of non-specifically bound RVV-XA. All complexes would be eluted from the anti-fluorescein absorbent and then bound to the hapten-specific absorbent(s) in series. These would then be tested for the presence of RVV-XA, and only in those cases where there was toxin which allowed the binding of both (hapten-labeled and RVV-XA-labeled) antibodies in the 'sandwich' would there be binding to the second, toxin-specific hapten. This approach would be of special value in the detection of toxin and the identification of toxin type by a 'color test'

format, which is certainly of value in cases where a yes/no value is highly diagnostic (presence or absence of disease-causing agents).

A similar objective can also be achieved by taking advantage of the filtration and separation of the anti-fluorescein absorbent, but rather than using a multiplicity of distinct haptens, using only two haptens with which all antibodies are labeled. In this case, the mixtures are kept separate from each other and passed through a physically distinct portion of the anti-fluorescein filter, then eluted and bound to the second filter via the other hapten. The position of the positive reaction would identify which toxin was present in the mixture. This has greater simplicity for preparation of conjugates and filters, but typically would use more sample for the analysis since all the reaction mixtures would require separate samples.

Measurement of anti-tumor reactivity

If a person produces antibodies to tumors, those antibodies are apparently not effective in preventing growth of the tumor. This could be because they are produced in very low amounts or the tumor releases antigen into the circulation which neutralizes them. In the previous work in the inventor's laboratory, it was found that anti-tumor antibody reactivity was apparently found on 'membrane fragments' isolated from the ascites fluids of ovarian cancer patients which could bind to cultured tumor cells or cells grown in 'nude' mice .

If one identifies a tumor-associated antigen which is immunogenic in the cancer patient, then it is possible to use the same technique as was used for the measurement of antibodies to botulinum toxin for measurement of tumor-specific reactivity. That is, antibodies prepared against the relevant antigen and labeled with hapten and detector molecules could be added, in the presence of serum from the cancer patient, to pure or partially purified antigen preparations from tumors. If there is anti-tumor reactivity, then this specific reactivity will be apparent when the complex is eluted using hapten and bound to anti-human IgG. Similarly, if a pure antigen is labeled with both hapten and RVV-XA, and complex with patients' serum is captured on anti-hapten absorbent, then the eluted complex would also be identifiable using binding to anti-human IgG.

Cancer antigens in this case would be those antigens which induce a response in a cancer patient that would be related to the course of disease. In general, antigens identified by immunizing animals and determining which antigens appear to be related to tumor development (*e.g.* beta hGC in choriocarcinoma, prostate-specific antigen and its complexes with specific inhibitors as a reflection of prostate cancer, CA125 and placental alkaline phosphatase in ovarian

cancer, CEA in colon cancer, alpha-fetoprotein in hepatoma *etc.*) would only be assayed in this way if they were also capable of eliciting an immune response in a cancer patient. Better candidates could, in fact, be identified by a modification of the current invention, if they were labeled either by labeled antibodies or directly, and patient sera were screened for antibody reaction according to the present invention.

The advantage of this analysis, in the case that antigens are identified to which the cancer patient responds in the course of development of tumors, could include monitoring the occurrence of tumors and the course of their development, thereby allowing therapy appropriate to tumor type. It is to be noted that in detection of exposure to infectious diseases, measurement of antibody responses are considered to be the most reliable index. This is not the current practice in cancer diagnostics, either because of the absence of specific antigens to which the patient is responsive, or due to the difficulty of detecting small amounts of antibody using current technology. If the latter is the case for any cancer, then the current invention will permit the direct measurement of anti-tumor reactivity. Kit components would be very similar to those above for measurement of antibodies to botulinum toxin, except for the labeled antibodies or antigens and the antigen provided with the kit.

Other infectious agents; measurement of antibodies.

Antibodies to other infectious agents could similarly be determined with modifications of the same protocol. For example, antigens of AIDS virus and hepatitis A, B and C labeled with RVV-XA and the haptens listed above could be combined into a single test system which could be used in blood banks to assess exposure to these antigens by a protocol identical to the above, with different reagent tubes but with the same absorbents and kit components. This applies to any other infectious agent, such as viruses, bacteria and the toxins they produce, in evaluation of the exposure of patients to the same. In such cases, acute exposure could be determined by measuring IgM response.

Measurement of other antigens by hapten transfer.

It is also apparent that any other antigen could be analyzed using the hapten transfer approach, with the same advantages of specific transfer (reduced background, higher specificity, ability to separate reactivity into multiple components). For these applications, the same kit components as above would be included, with a different specificity of the labeled antibodies. Examples of other toxins would be Staph enterotoxins, Shiga-like toxins produced by bacteria such as *E. coli* O157/H7, etc. Examples of other infectious agents would be surface antigens of

viruses or bacteria, etc. It is possible under the current invention to devise a series of filters which could identify any combination of antigens with reagents which differ significantly only in the specificity of the antibodies used.

Kits for the detection of DNA/RNA.

In this case, the general kit which could be made available for use of the current invention, would be one in which RVV-XA-streptavidin, anti-fluorescein coated solid phases, and anti-digoxigenin coated solid phases could be provided for use by laboratories wishing to apply this assay principle in hybridization reactions. Probes labeled with fluorescein, biotin and digoxigenin would be available to these laboratories from commercial sources, and could be employed to detect sequences directly in hybridization reactions, or after a limited number of cycles of polymerase chain reaction with specific probes. A preferred embodiment in the case of PCR generated sequences (DNA) or RT-PCR sequences (RNA), may be for the laboratory to prepare primers labeled with biotin and fluorescein, and an internal probe labeled with digoxigenin. In such a case, the product of PCR would be hybridized to the digoxigenin-labeled probe, be bound to anti-fluorescein, washed and labeled with RVV-XA-Streptavidin, washed and eluted with fluorescein-gelatin, and bound to anti-digoxigenin then measured by ELCA.

In the event that only two labels were used, i.e. fluorescein and biotin, the kit would be designed to minimize the non-specific reaction of RVV-XA-streptavidin with the solid phase by providing the two solid phases consisting of anti-fluorescein and biotinylated protein. The biotinylated protein would bind the eluted RVV-XA-streptavidin-DNA/RNA using one of the remaining three binding sites not used in its binding to the biotinylated probe. This could further simplify the detection of amount of complex formed, since the biotinylated protein to which RVV-XA-streptavidin-DNA/RNA was bound could be biotinylated fibrinogen, which could also serve as a solid phase substrate/matrix for the deposition of enzyme-labeled fibrin.

Kits for the detection of viable bacteria.

In this case, the principal unique components would be enzyme-labeled and hapten-labeled antibodies reactive with surface antigens of the bacteria. For example, if one wished to detect the bacterial strain *E. coli* O157/H7, this could be the antibodies labeled with RVV-XA and fluorescein. Capture matrices would also be provided consisting of either a microtiter plate or a highly porous filter labeled with anti-fluorescein (for specific trapping and elution of bacteria, a filter with a pore size of 2-5 microns should be used).

In this specific embodiment, there are two objectives which may be addressed in a kit designed for this bacterium using the hapten elution approach. That is, one may wish to specifically identify the *E. coli* O157/H7 and subsequently isolate it in viable form. This could be accomplished by using fluoresceinated antibody to the somatic (O) antigen and RVV-XA-antibody to the flagellar (H) antigen. The combination of reagents would bind the bacterium to the microtiter plate or filter containing anti-fluorescein. The presence of the bacterium could then be assessed by ELCA assay, and when the positive samples were identified, the bacteria could be eluted using sterile medium containing fluoresceinated gelatin. The assay kit used to measure the bacterium (ELCA substrate, etc.) would be composed of buffers that did not affect the viability of the organism, which is possible using current formulations. A preferred form of anti-fluorescein absorbent in this case would be a filter absorbent with large pore size. That is the case, since it would be preferred if the matrix used to trap the bacterium could be readily washed. It is likely that the bacterium would be identified in highly contaminated specimens such as feces and cultures of food extracts, and it would be desirable if large amounts of buffer could be used to wash the trapped bacteria before sterile fluorescein-gelatin containing medium were used to elute the bacteria.

The same approach can be used without significant modification for a wide variety of other bacteria and viruses. The variation in kits specific for various analytes would therefore be the fluoresceinated and RVV-XA-antibodies used to capture the bacterial complex and the growth medium for subsequently allowing the organism to grow. It may be the preferred embodiment to provide only the fluoresceinated gelatin or casein in a sterile, concentrated solution and assume that each laboratory would have a preferred growth medium.

Generally speaking, kits in accordance with the present invention will include, for antibody detection, an antigen composition, a suitable hapten conjugated antibody directed against the antigen, a labeled antibody directed against the antigen, such as a RVV-XA labeled horse antibody and a means for containing the various preparations. The components of the diagnostic kits may be packaged either in aqueous media or in lyophilized form. In addition, the kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, or as separate moieties to be conjugated by the user of the kit.

The kits of the present invention will generally comprise a container means, including within the container at least one vial, test tube, flask, bottle, syringe or other smaller container means, into which the antigens, antibodies or reagents may be placed, and preferably suitably

aliquoted. Where a second or further binding ligand is provided, the kit will also generally contain a second vial or other container into which this ligand or antibody may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers in close confinement for commercial sale. Such containers may include injection or blow-molded plastic containers into which the desired vials are retained.

* * *

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Material and Methods

In the previous sections and in other reports, the inventor has described the details of the development of the ELISA-ELCA assay for botulinum toxin (Doellgast *et al.*, 1993; Doellgast *et al.*, 1994a and 1994b; Roman *et al.*, 1994; Doellgast *et al.*, 1995) and for measurement of antibodies to toxin by competition for immune complex formation (Doellgast *et al.*, 1995). The reagents developed in those studies were used in the current work, with the following modifications:

Human sera from subjects immunized with pentavalent toxoid were from the Centers for Disease Control, and were principally subjects who contributed sera used in the preparation of the human antibody preparation currently being used for treatment of infant botulism (Botulism Immune Globulin, or BIG) and control subjects who had not been so immunized and who did not have measurable antibody by mouse neutralization. Sera from subjects being treated for spastic disorders by injection of botulinum toxin A (BOTOX) were patients at the Center for Voice Disorders, Bowman Gray School of Medicine and North Carolina Baptist Hospitals.

Horse antisera used for the preparation of labeled anti-botulinum toxin were from animals immunized for 3 years with preparations of toxoids from individual toxin serotypes, either A, B or E in this case and maintained in the Detrick large animal facility (Fort Detrick,

Frederick, MD). The immunoglobulin fraction was obtained from these antisera by precipitation in 40% saturated ammonium sulfate. An $(Fab')_2$ preparation was made from these precipitates by digestion of the immunoglobulin preparation with 2% by weight pepsin in 0.1 M acetate buffer, pH 4.0 overnight at room temperature. The $(Fab')_2$ fraction was purified on a 1 × 50 cm column of Sephacryl S200. Labeling of this fraction was as described previously (Doellgast *et al.*, 1995), using fluorescein maleimide and RVV-XA-maleimide derivatives. The derivatized fractions were used without further purification. It should be noted that the antibody fraction was not affinity purified, nor was it toxin-specific. The toxoids used for immunization were from toxin complex rather than pure neurotoxin. Specificity of the assay derives therefore from the use of toxin in the complex-specific immunoassay.

Monoclonal antibodies were raised against toxins A and B as previously described (Doellgast *et al.*, 1995). IgG fractions of mouse ascites were purified by ammonium sulfate precipitation and QAE-agarose chromatography as previously described (Doellgast *et al.*, 1995). Monoclonal antibodies were not digested by pepsin before use, but were reduced using 5 mM dithiothreitol and derivatized with fluorescein maleimide.

Protocol for the measurement of antibody titer using affinity elution.

Microtiter plate protocol

Mixtures of fluoresceinated horse polyclonal or mouse monoclonal antibody and RVV-XA-labeled horse antibody with various dilutions of either chicken immunoglobulin preparations or human serum were mixed with toxins and incubated in solution phase for 1-48 hours in Casein dilution solution (0.1 M Imidazole-HC1, 0.5 M NaC1, 0.5% Triton X-100, 10 mg/ml gelatin). The complexes were then bound to a microtiter plate coated with anti-fluorescein for 1 hour at 37°C or 16-24 hours at 4°C depending on the study. After binding, the plate was washed with saline using the Elcawash microtiter plate washer (Elcatech, Inc., Winston-Salem NC) and soaked with calcium-gelatin wash solution (0.025 M Imidazole-HC1, 0.15 M NaC1, 3 mM CaC12, 10 mg/ml bovine gelation, pH 7.6) for 10 minutes at room temperature. The plates were then washed with saline and incubated with 1 mM fluorescein in casein dilution solution containing 1 mg/ml non-specific horse IgG for 1 hour at 37°C unless otherwise stated. The solution in the microtiter well was then transferred into a plate coated either with anti-chicken IgY or anti-human IgG depending on the study, and incubated at 37°C for 60 minutes. The plate

was then washed with saline and soaked for 3 × 5-minutes intervals in calcium-gelatin wash solution, then assayed by ELCA as described previously (Doellgast *et al.*, 1993; Doellgast *et al.*, 1994; Roman *et al.*, 1994; Doellgast *et al.*, 1995). The protocol is presented above for human antibody. It was identical for chicken antibody, except that the eluted complex was bound to anti-chicken IgY.

Assay using anti-fluorescein gel

A pipet tip was loosely plugged with cotton. A suspension of 50 µl of anti-fluorescein beads were added to the mixture of 500 µl of fluoresceinated horse antibody, RVV-XA-horse antibody, toxin at concentrations of 0, 2, 20 and 200 ng/ml and 1 µg/ml of affinity-purified chicken antibody. After incubation overnight at 4°C, the mixture was collected in the plugged pipet tips, and washed thoroughly with buffer. 400 µl aliquots of 1 mM fluorescein in casein dilution buffer were passed through the column rapidly and the eluates were collected into tubes. The columns were then incubated an additional 30 minutes at room temperature and another 400 µl of 1 mM fluorescein was passed through and collected. The columns were then incubated an additional 150 minutes and washed through with another 400 µl of 1mM fluorescein. The eluates from each series were then serially diluted in 2-fold increments onto a plate of goat anti-chicken IgY in casein, incubated, washed and assayed.

EXAMPLE 2

Fluorescein Elution and Complex Binding

Toxin B was serially diluted from 10 ng/ml to 14 pg/ml in 3-fold increments and a mixture of F1-horse antibody, RVV-X-horse antibody, and affinity-purified chicken antibody at a concentration of 100 ng/ml was added. This mixture was allowed to incubate overnight and was then bound to plates of goat anti-fluorescein and goat anti-chicken IgY.

The goat anti-chicken IgY plate had a limit of detection of 40-120 pg/ml of toxin when both fluoresceinated horse antibody and affinity-purified chicken antibody were present in the complex. The goat anti-fluorescein plate, before assay, was treated with fluorescein concentrations of 0, 0.12, 0.37, 1.1, 3.3 and 10 mM for 20 hours and then the eluate was transferred to a plate of goat anti-chicken IgY, incubated for 60 minutes at 37°C, and both plates (anti-fluorescein, goat anti-chicken IgY) were assayed.

The detection limit of toxin on the two plates (Anti-fluorescein, anti-chicken IgY) is shown in Table 3. When the anti-fluorescein plate was not incubated with fluorescein, the detection limit of toxin was 43 pg/ml. After elution with fluorescein concentrations of 0.12-10 mM, the detection limit was significantly reduced on the anti-fluorescein plate, suggesting elution of the complex. Some of the complexes eluted from the anti-fluorescein plates were able to bind to the anti-chicken IgY plate, as indicated by the presence of measurable complex bound to the plate and the absence of such complex when fluorescein was not used to elute complex. The limit of detection appeared to be about 1 ng/ml of toxin present in the complex eluted from the anti-fluorescein plate under these conditions.

Table 3

Detection limit of toxin bound to goat anti-fluorescein plate after 20 hours of elution with fluorescein added at various concentrations and to goat anti-chicken IgY after 1 hour of binding of the eluted complex. Units are in ng/ml of toxin detectable on the two plates.

Plate	Elution with fluorescein at a concentration of:					
	0 mM	10 mM	3.33 mM	1.11 mM	037 mM	0.12mM
Goat anti-chicken IgY	>10	1.11	1.11	1.11	1.11	10
Goat anti-fluorescein	.043	0.37	0.37	0.12	0.12	0.12

EXAMPLE 3

Assay specificity; chicken antibodies. Fluoresceinated monoclonal and polyclonal antibody capture.

Using the affinity-purified chicken antibodies, the inventor utilized reaction mixtures consisting of fluoresceinated polyclonal (horse) and fluoresceinated monoclonal (mouse) antibodies for capture and RVV-XA-labeled polyclonal (horse) antibody. Affinity-purified chicken antibodies specific for either the A or B toxins were serially diluted into the antibody mixture, and toxin was added to a final concentration of 10 ng/ml. The mixture was incubated for 3 hours at 37°C on an anti-fluorescein capture plate, and the plate was then washed and eluted

with fluorescein overnight at 4°C. The eluate was transferred to a plate of goat anti-chicken IgY, washed and assayed.

The results are given in Table 4. The specificity of the reaction with toxins A and B was demonstrated with these affinity-purified antibodies, in that no reaction was found with the chicken antibodies against the heterologous antigen (either A or B). Sensitivity of detection reached a limit of 3.7 ng/ml using highly diluted fluoresceinated horse antibody in this study. Fluoresceinated monoclonal antibodies yielded a less sensitive assay, but with equal specificity.

Table 4

Assay of chicken antibodies using fluoresceinated monoclonal (FL-MC) and polyclonal (FL-PC) antibodies for capture of complexes. RVV-Ab and toxin used were the same specificity as the fluoresceinated antibodies. Chicken antibodies were tested for reaction with either toxin A or B. Value given is the limit of detection of the diluted chicken antibody under the given assay conditions.

	Mixture with specificity for A			Mixture with specificity for B		
Chick Ab to:	FL-PC-A 1:1.5 K	FL-PC-A 1:13.5 K	FL-MC-A 1:4.5 K	FL-PC-B 1:1.5 K	FL-PC-B 1:13.5 K	FL-MC-B 1:4.5 K
Toxin A	11 ng/ml	3.7 ng/ml	11 ng/ml	>100 ng/ml	>100 ng/ml	>100 ng/ml
Toxin B	>100 ng/ml	>100 ng/ml	>100 ng/ml	33 ng/ml	3.7 ng/ml	11 ng/ml

EXAMPLE 4

Detection of antibodies in crude preparations of chicken IgY

In order to evaluate the usefulness of the assay for measuring antibody titers in samples which contained specific antibody as a small fraction of the total immunoglobulin present, the inventor used IgY fractions from egg yolks of chickens immunized with toxoids prepared from toxins A, B and E. The results for this, expressed as relative dilutions, are shown in Table 5. The antibody specificity was that of the toxoid antigen, and no antibody against the heterologous antigen was found within the range of dilutions tested.

Table 5

Assay of chicken antibodies using fluoresceinated polyclonal antibodies for capture of complexes. RVV-XA-Ab and toxin used were the same specificity as the fluoresceinated antibodies. Chicken antibodies were from concentrated antibody preparations prepared by ion-exchange chromatography, in which the IgY concentrations were approximately 50-100 mg/ml. 1:100 dilutions, which were the highest concentrations tested, represent 0.5-1 mg/ml of IgY. Indicated is the maximal dilution of this preparation which gave measurable signal.

	Assay specific for:		
Chicken antibody to:	A	B	E
Toxin A	1:8100	<1:100	<1:100
Toxin B	<1:100	1:24300	<1:100
Toxin E	<1:100	<1:100	1:900

EXAMPLE 5

Use of anti-fluorescein beads; time for elution of complex using added fluorescein.

The binding of complex to a microtiter plate is limited by the capacity of the plate for absorption. A more effective approach for trapping complex is to bind to solid-phase matrices such as columns or magnetic beads coated with anti-fluorescein, since the total surface area for absorption is much larger. This approach was used, as described under Materials and Methods.

The results of this approach are shown in Table 6. Most of the detectable antibody was eluted from the anti-fluorescein columns within 30 minutes. Additional antibody was eluted at 150 minutes, but comparing the dilutions of complex detectable at this elution time suggests that less than 20% of the eluted complex was eluted after 30 minutes of treatment, since subsequent treatment of the columns for up to 2½ hours did not add appreciably to the total amount of antibody eluate. No signal was detectable when toxin was absent from the reaction mixture. Optimal sensitivity of the assay for detection of antibody was found in this study to be at the highest concentration of toxin (*i.e.*, 100 ng/ml).

Table 6

Measurement of antibodies eluted from anti-fluorescein beads. Results indicate the dilution of eluted complex which was measurable after binding to an anti-chicken IgY plate. 1 μg/ml of the affinity-purified chicken antibody was added to the reaction mixture (see Materials and Methods).

	Elution with fluorescein for (time)		
Reaction mixture:	**0**	**+30 minutes**	**+150 minutes**
Ab to A plus 2 ng/ml toxin A	1:2	<Undiluted	<Undiluted
Ab to A plus 20 ng/ml toxin A	1:32	1:16	1:4
Ab to A plus 200 ng/ml toxin A	1:32	1:32	1:16
Ab to B plus 2 ng/ml toxin B	1:32	1:16	1:2
Ab to B plus 20 ng/ml toxin B	1:128	1:64	1:32
Ab to B plus 200 ng/ml toxin B	>1:128	1:128	1:64
Ab to E plus 2 ng/ml toxin E	1:4	Undiluted	<Undiluted
Ab to E plus 20 ng/ml toxin E	1:32	1:16	1:2
Ab to E plus 200 ng/ml toxin E	1:64	1:32	1:8

Similar results were obtained in several studies on microtiter plates, indicating that 30-60 minutes of fluorescein elution was optimal.

EXAMPLE 6

Optimization of conditions for antibody assay.

The study presented in the following example used a single concentration of each specific antibody, (*i.e.*, 1 μg/ml). In other studies in which the antibodies were serially diluted the inventor found that optimal concentrations of toxins A, B and E for detection of diluted specific chicken antibody were 10, 100 and 20 ng/ml respectively. Using these concentrations of toxins, the inventor optimized the dilutions of fluoresceinated antibody and RVV-XA-Ab to yield maximal sensitivity of antibody detection. The results of assay of affinity-purified chicken antibodies is shown in FIG. 1. Antibody to toxin A was measurable at 1 ng/ml, that for toxin B at 0.4 ng/ml and that for toxin E at 4 ng/ml.

EXAMPLE 7

Test of human antibodies; individuals immunized with toxoid.

Using the F1- and RVV-XA-antibody dilutions of FIG. 1, the inventor tested human antisera against botulinum toxin obtained from individuals immunized with multivalent toxoid. Sera from non-immunized individuals gave no detectable antibody in this series when tested undiluted. Table 7 gives the results for 3 such sera tested against toxins A, B and E.

In these assays, titers as great as 8,100-24,300 were measurable in selected subjects. The wide variability of response to the different toxins present in the toxoid preparation is indicated by the low titer of antibody to toxin E in subject 1. Note that under these conditions the assay for affinity-purified chicken antibody to toxin E was less sensitive (detection limit 4 ng/ml) than that to toxin A (1 ng/ml) or B (0.4 ng/ml) (FIG. 2).

Table 7

Reaction of three toxoid-immunized individuals to toxins A, B and E. The values shown are the optical densities obtained in the ELCA assay. The value for titer is the last dilution at which an optical density >= 0.05 was obtained.

	Toxin A			Toxin B			Toxin C		
Dilution (fold)	1	2	3	1	2	3	1	2	3
100	0.95	1.45	1.25	1.46	1.87	1.07	0.12	1.6	1.39
300	1.10	1.40	1.32	1.78	1.76	1.22	0.05	1.25	1.50
900	0.75	1.19	1.25	1.44	1.63	1.08	0.02	0.34	1.05
2,700	0.26	0.65	1.24	1.02	1.09	1.02	0.01	0.07	0.25
8,100	0.06	0.25	0.14	0.09	0.19	0.58	0.01	0.02	0.04
24,300	0.01	0.08	0.15	0.01	0.03	0.13	0.02	0.01	0.02
72,900	0.00	0.05	0.02	0.06	0.02	0.02	0.01	0.00	0.01
TITER	8100	24300	24300	8100	8100	24300	100	2700	2700

EXAMPLE 8

Antibody in BOTOX-treated patients.

65 samples of sera from BOTOX-treated patients were tested for their reactivity against toxin A (the administered form) and toxin B (antisera against this are not reactive with the administered toxin). Of the 65 samples, four were reactive with toxin A, two of which were reactive to toxin B as well. 18 sera showed reactivity with toxin B. Five of the sera which were reactive with either toxin A or B are shown in Table 8.

In serum diluted 2-fold, the assay can discriminate between three individuals who produce antibody to toxin B and two who produce antibody against A. As controls, assays were performed with these 1:1 diluted sera using reaction mixtures without added toxin. In all cases, there was no detectable antibody measured by this technique in the absence of toxin. For the two samples showing reactivity with toxin A (samples 4 and 5), sample 5 showed reproducible, very low titer of antibody by this protocol. Sample 4, which was from a subject who was receiving very high doses of BOTOX for therapeutic reasons, was producing measurable amounts of antibody to toxin A. The three subjects producing antibody against toxin B showed very different titers. None of these samples had measurable antibody against toxin E.

Table 8

Reactions of sera from BOTOX-treated patients with botulinum toxins A and B. Assay conditions were identical to those used for Table 7 above; assays were performed on the same plates.

Assay for human antibody in five BOTOX-treated subjects; specificity for:

	Toxin A					Toxin B				
Dilution (fold)	1	2	3	4	5	1	2	3	4	5
2	0.00	0.06	0.02	0.83	0.48	1.07	1.03	0.70	0.03	0.03
4	0.01	0.05	0.00	1.07	0.02	1.43	0.77	0.22	0.03	0.03
8	0.01	0.06	0.02	0.88	0.01	1.41	0.41	0.09	0.03	0.01
16	0.03	0.02	0.00	1.31	-0.02	1.98	0.22	0.08	0.02	0.00
32	0.06	0.01	0.01	0.88	-0.01	1.75	0.20	0.03	0.01	0.00
64	0.01	0.06	0.02	0.80	-0.02	1.41	0.07	0.03	0.02	0.00
128	0.02	0.05	0.07	0.59	0.00	0.93	0.06	0.02	0.01	0.00
256	0.02	0.00	0.05	0.19	0.00	0.29	0.03	0.05	0.03	-0.01
TITER	None	None	None	>256	2	>256	64	16	None	None

EXAMPLE 8

Infant Botulism Study

The further demonstration of the utility of this approach has involved the testing of the method for measurement of antibodies to *C. botulinum* neurotoxin in cases where individuals are infected with this organism (infant botulism) and this toxin which are being studied for therapy using passive antibody administration. In cases of infant botulism, infants who accidentally ingest spores of *C. botulinum* will allow the organism to colonize the intestine and produce toxin which paralyzes the infant. These infants have been treated in the past by placing them on respirators in an intensive care nursery, and subsequently they almost invariably fully recover.

In a clinical protocol in California, these infants are being treated in a double-blind, placebo-controlled study by administration of a human antibody preparation which is reactive with botulinum toxin and consequently is able to neutralize the toxin in the infant's bloodstream. Since the protocol is placebo-controlled, half of the infants do not receive passively administered

anti-toxin antibodies. It is to be noted that infants typically are infected with strains of *Clostridium botulinum* which produce either toxin A or toxin B, though recent studies have suggested that both may be produced by some strains.

The inventor participated in this project, to the extent that the ELISA-ELCA hapten transfer method was developed initially to enable the measurement of antibody titers in this group of patients, with the goal of measuring the clearance rate of the injected antibody or the rate of development of the specific antibody in those patients receiving placebo controls. The project was recently completed, after enrolling the targeted number of patients, and the code has now been broken. The results to date demonstrate that the method can correctly identify the individuals who received placebo and it was found that these patients did develop specific antibodies. Table 9 and 10 show the results for several of these patients. It is apparent that the patients who received placebo reacted with the type of neurotoxin with which they were known to be infected, with the exception of Table 10, infant 7. In this case, there is not yet an explanation available for the reaction of this infant with both toxin types. The possibility of infection with both types from a single organism or mixture of organisms cannot be excluded.

Table 9

Test of infant botulism sera for reaction with toxins A and B. Sample I was from a control (placebo) infant infected with toxin B, sample II was from a placebo infant infected with toxin A, Sample III and IV were from infants receiving antibody to botulinum toxins A and B. The first sample in most cases was pre-infusion of the control or positive antibody. Values given in each case are as milli-international units using the infused antibody as a control for the assay.

I-A	II-A	III-A	IV-A	I-B	II-B	III-B	IV-B	Sample
0	15	0	0	0	0	0	63	1
0	10	833	250	0	0	80	208	1
0	63	333	125	5	0	30	208	2
0	125	250	169	417	0	10	200	3
0	31	167	63	417	0	5	167	4
0	25	167	50	250	0	4	125	5
0	15	83	31	167	0	5	75	6
0	13	42	16	92	0	2	42	7
0	10	25	4	75	0	1	33	8
0	0	6	4	42	0	0	21	9

Table 10

Test of infant botulism sera for reaction with toxins A and B. Results are separated into A and B reactivities. The first sample in most cases was pre-infusion of the control or positive antibody. This represents a different group of patients. Patients 1-3 and 8 were placebo babies infected with toxin A; 7 was a placebo baby thought to be infected with toxin B, but apparently also producing antibody to toxin A; Patients 4-6 were babies receiving specific antibody.

Part 1 - Reaction with neurotoxin A:

	I	II	III	IV	V	VI	VII	VIII
1	1	0	375	938	1	9	2	19
1	1	0	583	156	595	375	1	9
2	208	78	667	125	238	188	30	96
3	367	188	250	75	226	344	42	77
4	833	469	208	156	149	281	180	38
5	1000	313	25	125	179	250	300	48
6	667	141	50	19	60	281	180	17
7	383	5	13	2	89	131	160	19
8	333	8	10	0	71	109	130	19
9	250	6	0	625	24	125	120	3
10	13	8	33	8	30	63	25	8

Part 2 - Reaction with neurotoxin B.

	I	II	III	IV	V	VI	VII	VIII
1	0	0	0	50	0	0	0	0
1	0	0	0	40	50	96	0	1
2	0	0	0	28	24	38	0	0
3	0	0	0	24	28	31	1	0
4	0	0	0	16	20	15	38	0
5	0	0	0	12	16	12	46	1
6	0	1	0	6	12	8	42	1
7	0	6	0	3	26	4	19	0
8	0	0	0	0	10	3	12	0
9	0	1	0	60	16	1	10	0
10	0	0	0	0	6	0	0	0

EXAMPLE 9

Selectivity of Elution Using Fluoresceinated Proteins.

In this case, 2 sets of 96 - 100 µl columns were prepared using a commercially available monoclonal antibody against fluorescein, obtained from Sigma Chemical Company, St. Louis. (Product # 096H4804; mouse ascites fluid Clone FL-D6). This was coupled to Emphase

absorbent purchased from Pierce Chemical Company. The columns were prepared by drilling a 0.75 inch long, 0.125 inch diameter hole in a polycarbonate block with the same spacings as a microtiter plate (0.355 inches apart), then gluing in 0.125 inch OD tubing, using cotton as a 'plug', then filling with alternating layers of 25 μl of G-25 Sephadex, 25 μl of monoclonal anti-fluorescein, and filling with G-25 Sephadex. To these columns was added various dilutions of human sera reactive with both toxin A and B, mixed with fluoresceinated horse antibody, RVV-XA-labeled horse antibody, and toxin according to the current invention. After binding and washing the column, the columns were eluted with fluorescein, fluoresceinated gelatin or fluoresceinated casein diluted into elution buffer (10 mg/ml gelatin, 50 mM imidazole-HCl, 0.5 M NaCl, pH 7.0). The eluted samples were bound to anti-human IgG-coated microtiter plates and assayed by ELCA. Results for the assay are shown in Table 11 below, presented as optical density values (OD550-OD450, blank corrected):

Table 11

Elution with fluorescein and fluoresceyl-protein derivatives; assay of human anti-botulinum toxin.

Toxin A reaction Elution with Fluorescein				Fluoresceyl gelatin				Fluoresceyl casein				Diln
1 mM	0.33	0.11	0.037	1mM	0.33	0.11	0.037	1 mM	0.33	0.11	0.037	Serum
0.656	0.36	0.665	0.518	0.654	0.612	0.599	0.486	0.654	0.641	0.628	0.528	100
0.258	0.278	0.216	0.06	0.344	0.546	0.516	0.523	0.301	0.385	0.488	0.501	300
0.08	0.094	0.03	0.03	0.289	0.284	0.244	0.07	0.124	0.306	0.293	0.355	900
0.03	0.02	0.01	0.01	0.04	0.102	0.086	0.082	0.08	0.07	0.112	0.179	2700
0	0	0	-0.01	0.03	0.02	0.03	0.01	0.02	0.02	0.06	0.114	8100
0	0	-0.01	-0.01	0.113	0.04	0.02	0.01	0.03	0.02	0.04	0.082	24300
0	0	0	0	0.01	0.02	0.01	-0.01	0.01	0.01	0.03	0.06	72900
0.02	0	0	0.01	0.03	0.02	0.02	0	0.01	-0.02	0.02	0.08	218700

Toxin B reaction Elution with Fluorescein				Fluoresceyl gelatin				Fluoresceyl casein				Diln
1 mM	0.33	0.11	0.037	1mM	0.33	0.11	0.037	1 mM	0.33	0.11	0.037	Serum
0.601	0.518	0.715	0.534	0.772	0.754	0.75	0.686	0.507	0.583	0.57	0.702	100
0.398	0.394	0.277	0.04	0.469	0.434	0.48	0.333	0.271	0.308	0.37	0.568	300
0.222	0.226	0.189	0.08	0.272	0.367	0.182	0.279	0.138	0.159	0.174	0.419	900
0.163	0.109	0.05	0.01	0.164	0.179	0.274	0.02	0.07	0.07	0.142	0.194	2700
0.06	0.02	0.02	0.02	0.106	0.03	0.01	0.02	0.04	0.06	0.02	0.105	8100
0.01	0.01	0	0	0.04	0.03	0.04	0.02	0.05	0.03	0.03	0.05	24300
0.01	0.01	0.01	0	0.04	0.04	0.02	0.01	0.02	0.03	0.04	0.05	72900
0.01	0	0.01	0	0.05	0.04	0.02	0.01	0.04	0.08	0.04	0.04	218700

It is clear that, although the hapten fluorescein is effective at a concentration of 1 mM in elution of complexes, fluoresceinated gelatin and casein are more effective at a lower concentration. The decision to use either fluorescein or fluoresceinated proteins for elution may be made on other technical grounds such as convenience and relative toxicity of the two forms.

The fact that hapten is more effective for complex elution when bound to proteins is not surprising except in contrast to the report of Voss *et al* (1976) who proposed that there should be very little difference between fluorescein and its chemical conjugates. An additional reason for the greater efficiency of protein derivatives for this invention, is that the multiple binding sites available on a protein permits multi-site attachment of to the anti-hapten capture matrix, thereby having a greater strength of interaction, or 'avidity' of interaction in immunochemical terms.

Use of hapten-derivatized proteins also would substantially reduce the toxicity of elution buffers in the case where the objective was to elute viable bacteria from selected anti-hapten absorbents. The haptens in this case would be usable at a lower concentration and their association with a protein would in general reduce their potential toxicity since they would not freely enter the bacterial cell and would have to be digested before the hapten would be released. Preferred embodiments would be proteins which could be sterilized by autoclaving, which is true for both casein and gelatin derivatives.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Belich and Trowsdale, *Molecular Biology Reports, 21:53-56* ,1995.

Campbell *et al.*, *J. Mol. Biol.*, 180:1-19, 1984.

Crowther, "ELISA;Theory and Practice", *In Methods in Molecule Biology* Vol. 42; Publ. Humana Press; New Jersey, 1995.

Doellgast *et al*,. *J. Clinical Microbiology*, 31:2402-2409, 1993.

Doellgast *et al.*, *J. Clinical Microbiology, 32:*105-111, 1994a.

Doellgast *et al.*, *Journal of Clinical Microbiology*, 35:578-83, 1997.

Doellgast *et al.*, *Journal of Clinical Microbiology*, 32:851-953, 1994b.

Doellgast *et al.*, In "Molecular Approaches to Food Safety" (Eklund, M., Richard, J.L., and Mise, K., eds.) Chapter 6, pp 83-98 Alaken, Inc., Fort Collins, Colorado, 1995.

Engvall and Perlmann, *Immunochem.* 8:871-873, 1971.

Engvall *Methods Enzymol*, 70 (A) p419-39, 1980.

Engvall, *Lancet*, 2 (8000) p1410, 1976.

Engvall, *Med Biol.*, 55 (4) p193-200, 1977.

Gefter *et al.*, *Somatic Cell Genet.*, 3:231-236, 1977.

Goding, In: *Monoclonal Antibodies: Principles and Practice*, 2d ed., Academic Press, Orlando, Fla., pp. 60-61, and 71-74, 1986.

Gripenberg *et al.*, *Scand J Immunol.*, 7 (2) p151-7, 1978.

Gupta *et al.*, *Thrombosis & Haemostasis*, 70:963-969, 1993.

Harlow and Lane Eds., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, Cold Spring Harbor, New York, 1988.

Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976.

Kohler and Milstein, *Nature*, 256:495-497, 1975.

Makler *et al.*, *Transfusion*, 21 (3) p303-12, 1981.

Roantree, R.J. In: Kadis S, Weinbaum G, Ajl SJ (eds): Microbial Toxins, vol 5, p 18.Verlag Chemie GMBH, Weinheim, Germany, 1971.

Roman *et al*.,. *J. Food Protection*, 57:985-990, 1994.

Sarngadharan *et al.*, Princess Takamatsu Symp, 15 p301-8, 1984.

Stone *et al.*, *J. Clin. Micro.*, 32:1742-1749, 1994.
U. S. Patent No. 3,654,090
U. S. Patent No. 3,690,834
U. S. Patent No. 3,817,837
U. S. Patent No. 3,867,517
U. S. Patent No. 3,935,074
U. S. Patent No. 3,975,511
U. S. Patent No. 3,996,345
U. S. Patent No. 4,020,151
U. S. Patent No. 4,196,265
U. S. Patent No. 4,367,110
U. S. Patent No. 4,452,901
U. S. Patent No. 4,668,621
U. S. Patent No. 5,221,605
U. S. Patent No. 5,238,808
U. S. Patent No. 5,310,687
Voss *et al.*, *Immunochemistry,* 13:447-453, 1976.

CLAIMS:

1. A method of detecting an analyte in a sample comprising the steps of:
contacting said sample with a first agent conjugated to a detectable marker and a second agent conjugated to a hapten under conditions effective to form a complex comprising said analyte and said first and second agents, wherein each of said agents binds specifically to a separate site on said analyte;
contacting said complex with an anti-hapten antibody immunoreactive with said hapten and bound to a solid support;
separating said solid support from said sample to obtain a solid support fraction;
contacting said solid support fraction with said hapten to elute said complex from said anti-hapten antibody; and
detecting the eluted complex.

2. The method of claim 1, further comprising the step of capturing the eluted immunocomplex on a second solid support bound to an anti-Ig antibody prior to detecting the eluted immunocomplex.

3. The method of claim 1, wherein said analyte is a multivalent antigen and said agents are antibodies immunoreactive with separate sites on said antigen.

4. The method of claim 1, wherein said analyte is an antibody and at least one of said agents is an antigen.

5. The method of claim 1, wherein said analyte is a toxin.

6. The method of claim 1, wherein said analyte is a microorganism.

7. The method of claim 1, wherein said analyte is a virus particle.

8. The method of claim 1, wherein said analyte is a eukaryotic cell.

9. The method of claim 1, wherein said analyte comprises a receptor and at least one of said agents is a ligand for said receptor.

10. The method of claim 1, wherein said analyte is a nucleic acid molecule.

11. The method of claim 1, wherein said sample is a tissue sample or a biological fluid sample.

12. The method of claim 1, wherein said sample is a serum sample.

13. The method of claim 1, wherein said sample is an environmental sample.

14. The method of claim 1, wherein said sample contains a plurality of analytes to be detected and wherein said sample is contacted with a plurality of first and second agents that each bind specifically to one or more of said analytes.

15. The method of claim 14, wherein each of said first agents is conjugated to a separately detectable marker.

16. The method of claim 14, wherein each of said second agents is conjugated to a different hapten.

17. The method of claim 16, wherein a second or subsequent hapten is used to elute a second or subsequent analyte to be detected from said solid support.

18. A method of concentrating an antibody in a sample comprising the steps of:
contacting said sample with:
a multivalent antigen immunoreactive with said antibody;
a hapten-conjugated antibody immunoreactive with said antigen;
a second antibody conjugated to a detectable marker and
an anti-hapten antibody immunoreactive with said hapten and bound to a solid support;
under conditions effective to form an immunocomplex including the antigen, the hapten-conjugated antibody, a second antibody conjugated to detectable marker and the anti-hapten antibody, and further comprising the steps of:
separating said solid support from said sample to obtain a solid support fraction;
contacting said solid support fraction with hapten to elute said immunocomplex from said anti-hapten antibody; and
capturing said eluted immunocomplex on a second solid support to obtain a concentrated antibody complex.

19. The method of claim 18, further comprising the steps of:
separating said second solid support to obtain a second solid support fraction; and
separating said concentrated antibody complex from said second solid support fraction.

20. A method of isolating an antigen from a solution comprising the steps of:
contacting said solution with a hapten labeled antibody that immunoreacts with said antigen to form an immunocomplex that comprises said antigen and said hapten labeled antibody;

capturing said immunocomplex on a solid support bound to an anti-hapten antibody that immunoreacts with the hapten label;

separating said solid support from said solution; and

eluting said immunocomplex from said solid support by contacting said immunocomplex with the hapten.

21. The method of claim 20, further comprising contacting said antigen with an antibody conjugated to a detectable marker, wherein said antibody is immunoreactive with a site on said antigen separate from the hapten labeled antibody, and wherein said immunocomplex comprises said antibody conjugated to a detectable marker.

22. The method of claim 21, further comprising the step of detecting said immunocomplex by detecting said detectable marker.

23. The method of claim 22, wherein said detecting is by coagulation assay of Russell's viper venom factor X assay.

24. The method of claim 20, wherein said antigen is a bacterial surface antigen and said immunocomplex comprises a viable bacteria.

25. The method of claims 1, 18 or 20, wherein said hapten is fluorescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red or o-phthaldehyde.

26. A method of detecting a nucleic acid molecule having a specific sequence comprising the steps of:

contacting said nucleic acid molecule with:

a first nucleic acid sequence complementary to said nucleic acid molecule labeled with biotin; and

a second nucleic acid sequence complementary to said nucleic acid molecule at a region of the nucleic acid distinct from the first complementary nucleic acid labeled with a hapten;

under conditions effective to form a complex comprising said first nucleic acid sequence and said second complementary nucleic acid sequence bound to their respective complementary sites on said nucleic acid molecule;

binding said complex to an anti-hapten antibody bound to a solid support;

washing said solid support;

contacting said complex bound to said solid support with streptavidin bound to a detecting means to form a streptavidin-labeled complex;

eluting said streptavidin-labeled complex with an excess of hapten;

binding said complex to a solid support bound with biotin; and

detecting said complex with said detecting means.

27. The method of claim 26, wherein said detecting means is Russell's viper venom factor X assay and said solid support bound with biotin further includes fibrinogen.

28. The method of claim 26, wherein said hapten is fluorescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red or o-phthaldehyde.

29. The method of claim 28, wherein said hapten is fluorescein.

30. A method of detecting a nucleic acid molecule having a specific sequence comprising the steps of:

contacting the nucleic acid molecule with

a first nucleic acid sequence complementary to said nucleic acid molecule labeled with biotin;

a second nucleic acid sequence complementary to said nucleic acid molecule at a region of the nucleic acid molecule distinct from the first complementary nucleic acid labeled with a hapten; and

a third nucleic acid sequence complementary to said nucleic acid molecule at a region of the nucleic acid molecule distinct from said first and said second complementary nucleic acid sequences labeled with digoxigenin,

under conditions effective to form a complex comprising said first nucleic acid sequence, said second complementary nucleic acid sequence and said third complementary nucleic acid sequence bound to their respective complementary site on said nucleic acid molecule;

binding said complex to an anti-hapten antibody bound to a solid support;

washing said solid support;

contacting said complex bound to said solid support with streptavidin bound to a detecting means to form a streptavidin-labeled complex;

eluting said streptavidin-labeled complex with an excess of hapten;

binding said complex to a solid support bound with anti-digoxigenin antibody; and

detecting said complex with said detecting means.

31. The method of claim 30, wherein said detecting means is Russell's viper venom factor X assay.

32. The method of claim 30, wherein said hapten is fluorescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red or o-phthaldehyde.

33. The method of claim 32, wherein said hapten is fluorescein.

34. A method of detecting an anti-botulinum toxin antibody in a sample comprising the steps of:

contacting said sample with:

a composition comprising a first antibody conjugated to a detectable marker;

a composition comprising a second antibody conjugated to a hapten; and

a third composition comprising a botulinum toxin;

under conditions effective to form a complex comprising said first antibody, said second antibody, and said anti-botulinum toxin antibody each binding to a separate site on said botulinum toxin;

contacting said complex with an anti-hapten antibody immunoreactive with said hapten wherein said anti-hapten antibody is bound to a solid support;

separating said solid support from said sample to obtain a solid support fraction;

contacting said solid support fraction with said hapten to elute said complex from said anti-hapten antibody; and

detecting said eluted complex.

35. The method of claim 34, wherein said botulinum toxin is selected from the group consisting of botulinum neurotoxin A, botulinum neurotoxin B and botulinum neurotoxin E.

36. The method of claim 34, wherein said hapten is selected from the group consisting of fluorescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red and o-phthaldehyde.

37. The method of claim 34, wherein said first antibody is conjugated to Russell's viper venom factor X activator.

38. The method of claim 37 wherein said detecting is by coagulation assay of Russell's viper venom factor X assay.

39. A method of isolating viable microorganism in a sample comprising the steps of:

contacting said sample with a first antibody conjugated to a hapten and a second antibody that is labeled with an enzyme, wherein said first antibody and said second antibody are immunoreactive with separate surface antigens on said microorganism, under conditions to allow a complex to form between said microorganism and said first and second antibodies;

contacting said complex with an anti-hapten antibody immunoreactive with said hapten wherein said anti-hapten antibody is bound to a solid support;

separating said solid support from said sample to obtain a solid support fraction;

identifying the presence of the microorganism; and

eluting said microorganism from said anti-hapten antibody bound complex.

40. The method of claim 39, wherein said microorganism is a bacterium.

41. The method of claim 39, wherein said microorganism is a virus.

42. The method of claim 39, wherein said bacterium is *E. coli* O157/H7.

43. The method of claim 42, wherein said first antibody is immunoreactive with flagellar (H) antigen.

44. The method of claim 42, wherein said second antibody is immunoreactive with somatic (O) antigen.

45. The method of claim 39, wherein said hapten is selected from the group consisting of fluorescein, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, texas red and o-phthaldehyde.

46. The method of claim 39, wherein said enzyme is Russell's viper venom factor X activator.

47. The method of claim 46, wherein said wherein said identifying is by coagulation assay of Russell's viper venom factor X assay.

48. The method of claim 39, wherein said eluting comprises contacting said complex with sterile medium containing hapten.

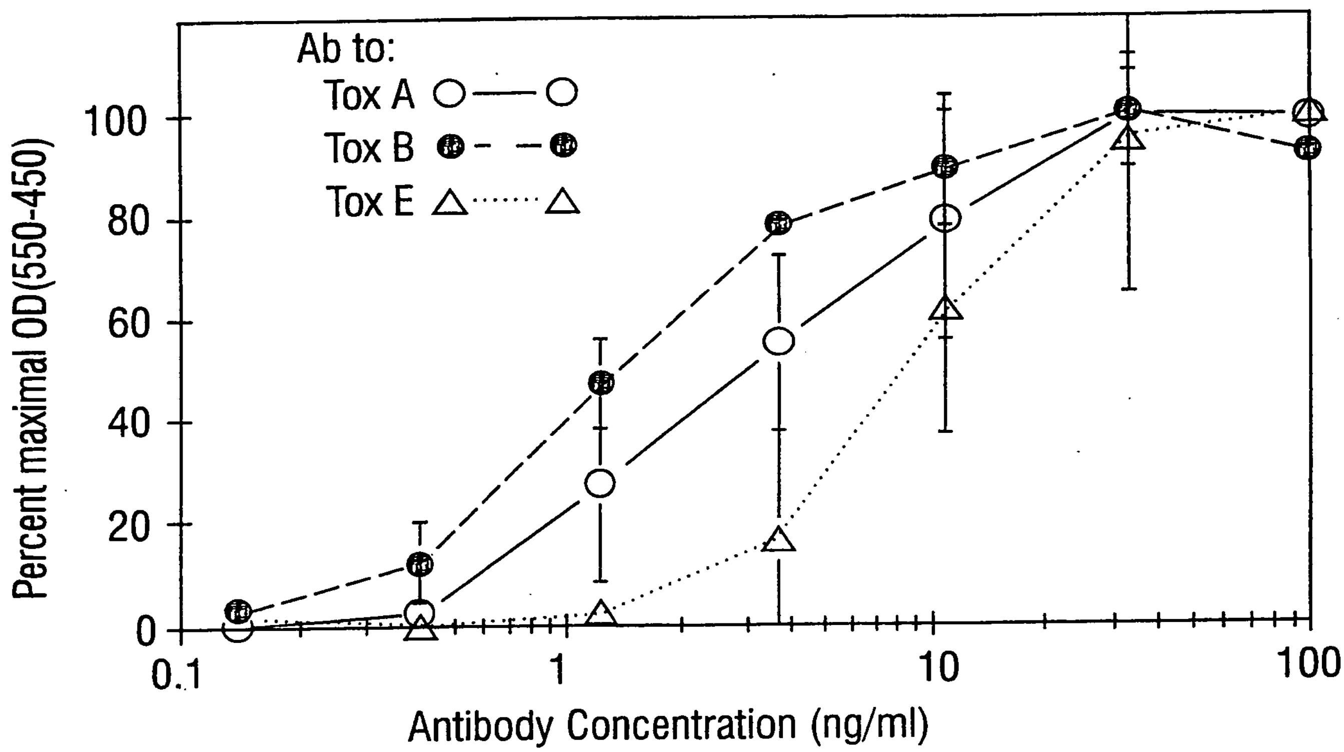
Ab to:
Tox A
Tox B
Tox E
Percent maximal OD(550-450)
0
20
40
60
80
100
0.1
1
10
100
Antibody Concentration (ng/ml)

FIG.1

STEP 1

Fi-Ab+RVV-Ab+Hu-Ab+Toxin ----→ Fl-Ab*Toxin*RVV-Ab *Hu-Ab

STEP 2

Fl-Ab*Toxin*RVV-Ab *Hu-Ab + Microtiter plate anti-Fl(MP-AF) ----→

MP-AF*Fl-Ab*Toxin*RVV-Ab *Hu-Ab

STEP3

MP-AF*Fl-Ab*Toxin*RVV-Ab *Hu-Ab $\xrightarrow{\text{1mM Fl}}$ MP-AF*Fl+ Fl-Ab*Toxin*RVV-Ab *Hu-Ab

STEP4

MP anti-IgG(MP-aIgG)+ Fl-Ab*Toxin*RVV-Ab *Hu-Ab

Fl-Ab*Toxin*RVV-Ab *Hu-Ab*MP-aIgG

STEP 5

Wash plate: use ELCA assay to detect complex bound to the anti-human IgG plate

FIG.2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19563

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/70
US CL :435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 5, 6, 7.2, 7.21, 7.25, 7.3, 7.32, 7.5, 7.7, 7.9, 7.92, 7.94, 243, 971, 973; 436/501, 518, 538, 543, 63, 69, 815, 822

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS
hapten and (anti(w) hapten) and (elute# or elution)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5,236,849 A (ISHIKAWA) 17 August 1993, see entire document, especially column 1, lines 9-24; column 2, lines 5-18 and 34-54; column 3, lines 1-16, 35-40, and 54-58; column 4, lines 47-60; column 5, lines 14-25; column 9, lines 14-51; column 12, line 38 to column 13, line 3; and claims 1-22.	1-4, 6-9, 11-12, 20-22, 24, 39-41 5, 10, 13-19, 23, 25-38, 42-48
Y	DOELLGAST ET AL. Sensitive Enzyme-Linked Immunosorbent Assay for Detection of Clostridium botulinum Neurotoxins A, B, and E Using Signal Amplification via Enzyme-Linked Coagulation Assay. Journal of Clinical Microbiology. September 1993. Vol 31. No. 9. pages 2402-2409, see entire document.	5, 10, 23, 27, 31, 46-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

24 JANUARY 1998

Date of mailing of the international search report

20 FEB 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19563

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,354,657 (HOLTKE et al.) 11 October 1994, see entire document, especially column 1, lines 10-19; column 2, lines 39-62; column 3, lines 22-48; column 4, lines 27-38 and 44-49; and column 5, lines 15-28.	10, 26-29
Y	US 4,923,819 (FERNANDEZ et al.) 08 May 1990, see entire document, especially claim 5.	14-17
Y	DOELLGAST ET AL. Enzyme-Linked Immunosorbent Assay - Enzyme-Linked Coagulation Assay for Detection of Antibodies to Clostridium botulinum Neurotoxins A, B, and E and Solution-Phase Complexes Journal of Clinical Microbiology. March 1994. Vol. 32, No. 3. pages 851-853, see entire document.	34-38